1971

Properties of the ribosomes and ribonucleic acids of Mycoplasma hominis

Jerry Dana Johnson
Iowa State University

JOHNSON, Jerry Dana, 1944-

PROPERTIES OF THE RIBOSOMES AND RIBONUCLEIC ACIDS OF MYCOPLASMA HOMINIS.

Iowa State University, Ph.D., 1971
Biochemistry

Properties of the ribosomes and ribonucleic acids of *Mycoplasma hominis*

by

Jerry Dana Johnson

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1971

TABLE OF CONTENTS

ABBREVIATIONS AND SYMBOLS

A	adenosine
Å	Angstrom = 10^{-8} cm
AMP	adenosine-3'-phosphate
A_{260} unit	the amount of material which in a volume of 1 ml will give an absorbance of 1.0 at 260 nm when measured in a cell of 1 cm path length
C	cytidine
Ci	curie - unit of radioactivity equal to $3.70 \cdot 10^{10}$ disintegrations per second
CMP	cytidine-3'-phosphate
6,6-diMeA	6,6-N-dimethyladenosine
2,2-diMeG	2,2-N-dimethylguanosine
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
5FU	5-fluorouridine
g	acceleration due to gravity 980 cm/sec^2
G	guanosine
GMP	guanosine-3'-phosphate
h	hour
H_2U	5,6-dihydrouridine
I	inosine
IPA	N^6-(Δ^2-isopentenyl)adenosine
M	concentration in moles per liter
mA	milliampere

1MeA	1-methyladenosine
6MeA	6-N-methyladenosine
5MeC	5-methylycytidine
1MeG	1-methylguanosine
2MeG	2-N-methylguanosine
7MeG	7-methylguanosine
7MePu	unspecified purine nucleosides methylated in the 7 position
3MeU	3-methyluridine
mg	milligram
min	minute
ml	milliliter
mM	concentration in millimoles per liter
mRNA	messenger ribonucleic acid
MW	molecular weight in daltons
nm	nanometer
PPLO	pleuropneumonia-like organisms
psi	pounds per square inch
Ψ	pseudouridine
RNA	ribonucleic acids
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
rT	ribothymidine
S	Svedberg unit
SDS	sodium dodecylsulfate

TLC	thin-layer chromatography
TM-2	0.01 M Tris·HCl buffer (pH 7.8) containing 0.01 M $Mg(C_2H_3O_2)_2$
tRNA	transfer ribonucleic acid
4TU	4-thiouridine
U	uridine
µg	microgram
UMP	uridine-3'-phosphate
UV	ultraviolet

INTRODUCTION

Protein biosynthesis has been studied extensively in many laboratories and a great deal of information has been accumulated regarding the mechanism by which cells carry out this process (see review by Lengyel and Söll, 1969). However, the specific functions of most of the macromolecules involved in the synthesis of a protein have not been completely elucidated. This lack of precise knowledge regarding specific functions and structure-function relationships is due at least in part to the extreme complexity of the protein synthesizing machinery in all organisms which have been studied. In an attempt to identify a protein synthesizing system that is significantly simpler, and therefore more amenable to investigation, than any currently being studied, the ribosomes and RNA from a species of mycoplasma, M. hominis, have been characterized. This organism was chosen because its low genetic capacity, one of the smallest known for a free-living organism, places a very stringent limit on the possible complexity of the cell (Morowitz, et al., 1967).

Investigations involving both prokaryotic and eukaryotic organisms have identified a large number of cellular components that are directly involved in the biosynthesis of proteins. These include 40-60 species of tRNA and their cognate aminoacyl-tRNA ligases, the ribosomes composed of 50-60 different proteins in combination with 3 or 4 varieties of RNA, mRNA containing the information required to specify the amino acid sequence of each polypeptide, and a number of soluble proteins several of which participate at each step in the synthesis of the peptide chain: initiation, elongation, and termination. In addition to

these macromolecular constituents, the process has also been shown to require ATP, GTP, amino acids, Mg^{2+}, and K^{+} or NH_4^{+}.

Of the components required for protein biosynthesis, the ribosomes and tRNA are the most complex and provide the greatest challenge in determining structure-function relationships. For this reason, the work with Mycoplasma hominis has concentrated on these constituents. In order to place these results in proper perspective, a brief review of what is presently known about the structure and functions of ribosomes and tRNA is presented here.

The ribosomes are cell organelles consisting of RNA and protein on which the series of reactions required in protein biosynthesis occur. There are three principal classes of these particles which can be differentiated on the basis of their size and origin. These are the 55-60S particles associated with the mitochondria of eukaryotic cells (Brega and Vesco, 1971; Attardi and Ojala, 1971), the 70S form from prokaryotes, and 80S ribosomes which are found in the cytoplasm of eukaryotes (Spirin and Gavrilova, 1969). All ribosomes contain several species of RNA and a large number of proteins; all also consist of two unequal subunits. The 70S bacterial ribosomes, especially those of E. coli, have been investigated most extensively and their properties will be discussed as an example. All data are from Spirin and Gavrilova (1969) except where otherwise noted. The 70S monosome (MW $2.8 \cdot 10^6$) is composed of one 30S subunit (MW $1.0 \cdot 10^6$) and one 50S subunit (MW $1.8 \cdot 10^6$). The 30S particle contains a single molecule of 16S RNA (MW $0.55 \cdot 10^6$) and 21 proteins, all different from each other and those found in the 50S subunit, (Hardy, et

al., 1969). The RNA complement of the 50S subunit includes one molecule of 23S RNA (MW $1.1 \cdot 10^6$) and a molecule of 5S RNA (MW $4 \cdot 10^4$). The large subunit of mitochondrial ribosomes appears to lack this low MW RNA (Lizardi and Luck, 1971). In the 80S ribosomes of eukaryotic cells, the larger subunit contains, in addition to the 5S RNA, a molecule of 7S RNA (MW 4.5×10^4) hydrogen bonded to the high MW ribosomal RNA component (Pene, et al., 1968). The protein complement of the 50S subunit of bacterial ribosomes includes about 35 unique components. Altogether, there are about 55 different polypeptide chains in each 70S ribosome (Traut, et al., 1969). The specific function of each of these protein components is as yet unknown although progress is being made in studying this problem, at least for the 30S subunit of E. coli ribosomes, by reconstituting these particles from the isolated RNA and protein constituents (Nomura, et al., 1969).

The multiple forms of tRNA which function as adaptors in the translation of the genetic code comprise another complex component of the protein synthesizing machinery. The following summary of the properties of these molecules is, except where otherwise noted, taken from Zachau (1969). There are 40-60 different species of this type of RNA present in a cell. Their size ranges from 75-85 nucleotides (MW $2.5\text{-}3.0 \cdot 10^4$). Ten to twenty percent of the nucleotides in tRNA are modified or unusual forms of the four major components, G, C, A, and U. These include Ψ, H_2U, rT, IPA, and 4TU, as well as a variety of derivatives formed by methylations of the common nucleosides. Since there are no base pairing schemes which can account for the insertion of unusual nucleotides at specific positions

in an RNA chain during its synthesis, the assumption has been made that these components are formed by modification at the polynucleotide level. Evidence to support this hypothesis has been obtained in the case of a number of constituents. For example, several enzymes have been isolated that are capable of introducing methyl groups into tRNA molecules from special mutants that produce "submethylated" RNA (Starr and Sells, 1969; Björk and Isaksson, 1970). There are, however, several minor components of tRNA, including H_2U and Ψ which appear in all known tRNA molecules, for which there is no direct evidence regarding the mode of their biosynthesis. Rapid progress is being made in determining the nucleotide sequences of purified individual tRNA species, and a number of sequence regularities have become apparent. It has been possible to arrange the primary structures of all tRNA molecules that have been sequenced thus far into a clover leaf structure which maximizes intrachain base pairing. This structure contains four characteristic loops and a stem region. It is clear that amino acids are attached to the tRNA at the base of the stem through an ester linkage with the ribose moiety of 3' terminal adenosine. This nucleoside forms part of a sequence, CpCpA, common to a 3' terminus of all tRNA molecules. The loop found closest to the 3' terminus of the molecule is identified by the presence of another common sequence, GprTp ΨpC, found at a constant position, residues 21-24 from the amino acid acceptor end, in all tRNA molecules which have been sequenced. A loop of variable size, from 5 to 14 nucleotide residues, begins at the 5' end of the GprTp ΨpC loop. The portion of the tRNA molecule on either side of the residues forming the anticodon triplet, that region which interacts with mRNA, are also thought to be based paired to

form a loop. Another base paired region, beginning about 9 nucleotides from the 5' terminus of the polynucleotide, forms an additional loop which is characterized by an abundance of the modified nucleoside H_2U. It is not clear how the structures just described are involved in the various functions of tRNA. For example, structural elements forming specific aminoacyl-tRNA ligase recognition sites and non-specific ribosome binding sites must exist on all tRNA molecules, however they have not yet been identified. Also, the functions of the many modified nucleosides commonly found in tRNA remain unknown. The identification of the structure-function relationships which exist in tRNA remains one of the most interesting problems in nucleic acid biochemistry (Zachau, 1969).

Attempts to study specific functions of the many individual components in the complex systems just described have been made difficult by the experimental problems involved in the fractionation and subsequent reconstitution of these components, thus many important features of the vital roles the ribosomes and tRNA play in the process of translating the genetic code remain obscure. If a protein synthesizing system significantly simpler than those observed in the commonly studied eukaryotic and prokaryotic systems could be identified, these experimental difficulties would be reduced. To this end, a detailed examination of the RNA and ribosomes from one species of mycoplasma, M. hominis strain 4330, has been carried out. The genus Mycoplasma (PPLO) contains the smallest known free-living organisms. In addition to being physically diminutive, the genome size of some

species of this very diverse genus is only one-quarter to one-fifth that of E. coli (Morowitz, et al., 1967). It has been postulated that this amount of DNA approaches the minimum necessary to code for sufficient functions to sustain an autonomous, self-replicating organism (Morowitz, 1966). If the genetic capacity of the mycoplasmas does approach a minimum limit, this should be reflected in a reduction in the morphological and biochemical complexity of the cell. Evidence to support this hypothesis can be found in studies of the nutritional requirements of the mycoplasmas (Rodwell, 1969). It has been found that, as a group, these organisms are fastidious to the extent that only one species, M. laidlawii, has ever been grown on a chemically defined medium. Most of the organisms in this genus grow only on complex media showing absolute requirements for both sterols and lipid-protein supplements. At the ultrastructural level, studies by Maniloff, et al. (1965) and Maniloff (1969) have revealed that the mycoplasma possess a very simple intracellular morphology. The only structural elements visible at a resolution of 100 Å were a limiting membrane, DNA, and ribosomes. An indication of simplification at the molecular level comes from the work of Hall, et al. (1967) on the nucleotide composition of RNA from M. pulmonis strain 880. They found that the RNA of this organism either lacks or has extremely low levels of 2-O-methylribonucleosides, pseudouridine, 5-methyluridine (rT), and methylated guanosine derivatives, possibly because of the paucity of genetic information to code for the enzymes necessary to synthesize these components. These results have recently been confirmed and extended by several investigators (Hayashi, et al., 1969; Johnson, et al., 1970). These workers have characterized a species

of $tRNA^{Ile}$ from M. species (Kid) which lacks 5-methyluracil (ribothymidine), a modified nucleoside present at a specific position in the GprTp $^{\Psi}$pC loop in all other tRNA molecules which have been studied (Zachau, 1969). As discussed previously, the function of modified nucleosides in tRNA is an important problem in the study of nucleic acids and, tRNA species which lack either some or all of these constituents are very useful as tools in investigating the problem. The reports by Hall, et al. (1967) indicating that mycoplasma were a potential source of such tRNA species suggested a detailed study of this aspect of the protein synthesizing machinery from M. hominis. For this reason, an analytical technique capable of measuring very low levels of nucleosides, as little as 10-20 picomoles, was adapted for use with a number of the modified nucleosides commonly found in tRNA (see Appendix).

Few other studies on the apparatus of protein biosynthesis and the RNA of mycoplasmas have been reported. Tourtellotte (1969) has demonstrated amino acid activation and polypeptide formation in crude preparations derived from several species of mycoplasma. Kirk and Morowitz (1969) have studied the ribonucleic acids of M. gallisepticum, a species whose genome contains a relatively large amount of DNA, $1.2 \cdot 10^9$ daltons, compared to some of the smaller species of mycoplasma (Morowitz, et al., 1967). A very recent report by Feldmann and Falter (1971) describes some properties of the tRNA from M. laidlawii A.

The possibility that the protein synthesizing system in Mycoplasma might be significantly simpler than similar systems in other organisms has prompted a characterization of the RNA and ribosomes of one species of mycoplasma, M. hominis strain 4330. This species was chosen because

it has a very small genome, $4\text{-}5 \cdot 10^8$ daltons of DNA (Morowitz, et al., 1967), with a G + C content of only 27-29% (Kelton and Mandel, 1969).

EXPERIMENTAL PROCEDURE

Cell Growth and Media

Mycoplasma hominis type 1 (strain 4330), kindly supplied by Dr. William Kelton (National Animal Disease Laboratory, Ames, Iowa), was grown in flasks incubated at 37°, with aeration by shaking. The growth medium contained 2.5% heart infusion broth, 1% yeast extract, and 1% proteose peptone No. 3. This mixture was autoclaved, after the pH had been adjusted to 8.0-8.2 with NaOH, and then supplemented with PPLO serum fraction to a final concentration of 1%. Each liter of medium was innoculated with 10 ml of 48 h culture grown in the same broth. Cell growth was followed by measuring turbidity at 650 nm in a Bausch and Lomb Spectronic 20. The cells were harvested in late log phase by centrifugation and washed twice in buffer containing 10 mM Tris·HCl (pH 7.8), 10 mM $Mg(C_2H_3O_2)_2$, 60 mM KCl, and 1.0 mM dithiothreitol (standard buffer). Cells not used immediately were stored as a frozen paste at -20°. The yield was approximately 0.5 grams of cells per liter of medium.

E. coli strain B, grown to 3/4 log phase on an enriched medium, was purchased as a frozen paste from Grain Processing Corp., Muscatine, Iowa. Radioactively labeled ribosomes and RNA were prepared from cells of *E. coli* B grown on a glucose-salts medium (Demerec and Cahn, 1953) supplemented with 25 μCi/l of [2-^{14}C]uracil (22 mCi/mmole) or 250 μCi/l of [5-^{3}H]uridine (28.3 Ci/mmole). When the cultures were in mid log phase, the cells were harvested by centrifugation, washed with TM-2 buffer, and frozen at -20° until needed.

Preparation of Ribosomes

Ribosomes were prepared by the same procedure from both Mycoplasma and E. coli. All operations were performed at 0-4°. Cells, suspended in standard buffer containing 1-2 μg/ml DNase, were broken in a chilled French pressure cell at 10,000-12,000 psi. Unbroken cells and membrane fragments were removed by centrifugation at 25,000 x g for 45 min and the supernatant was then centrifuged for 4 h at 85,000 x g in the 40 rotor of the Spinco Model L4 ultracentrifuge to pellet the ribosomes. These were then resuspended in standard buffer and purified by two additional cycles of differential centrifugation. In some preparations, the final wash was made in standard buffer containing 0.5 M NH_4Cl in place of KCl. The ribosomes were then resuspended in TM-2 buffer, dialyzed against this buffer to remove residual NH_4Cl and, if not used immediately, frozen and stored at -85°.

For preparation of ribosomal subunits, ribosomes were dissociated by dialysis against 10 mM Tris·HCl (pH 7.8) containing 0.5 mM $Mg(C_2H_3O_2)_2$. These ribosomes (20 mg) were layered on a 5-20% linear sucrose gradient (Britten and Roberts, 1960) prepared in the same buffer and then centrifuged for 6.5 h at 25,000 rev./min in the SW25.2 rotor of the Spinco model L2 ultracentrifuge. Fractions of 1.4 ml were collected, either by puncturing the bottom of the centrifuge tube or with a Buchler auto Densi-Flow, and their absorbance at 260 nm measured. After pooling the appropriate fractions, the subunits were recovered by centrifugation at 85,000 x g for 20 h in the 40 rotor of a Spinco model L4 ultracentrifuge. The pellets were resuspended in 10 mM Tris·HCl containing

0.5 mM $Mg(C_2H_3O_2)_2$ and the subunits further purified by a second cycle of sucrose density gradient centrifugation. The resulting 30S and 50S preparations were 95-97% homogeneous as judged by the patterns observed in the analytical ultracentrifuge.

Extraction of RNA

M. hominis RNA was prepared directly from crude extracts. Cells were broken as described for the preparation of ribosomes except that bentonite (Fraenkel-Conrat, et al., 1961) at a concentration of 4-5 mg/ml, was added to the cell suspension before it was passed through the French pressure cell. The crude extract was immediately centrifuged at 25,000 x g for 45 min to remove unbroken cells, membrane fragments, and bentonite. Rapid removal of the membrane fraction with its associated ribonuclease activity (Pollack, et al., 1965) was a critical factor in preparing intact RNA from M. hominis. RNA was extracted from the supernatant using the phenol-SDS method described by Kurland (1960) modified by the addition of bentonite at a final concentration of 3-5 mg/ml. Phenol used for RNA preparations was vacuum distilled to remove impurities and stored at 4^{o} until used. The RNA was dissolved in different buffers as described in individual experiments. Transfer RNA preparations whose amino acid accepting activities were to be determined were first stripped of amino acids by incubation at 37^{o} in 1.8 M Tris·HCl (pH 8.0) for 90 min (Sarin and Zamecnik, 1964). E. coli RNA was prepared as described for Mycoplasma. In several experiments a commercial tRNA preparation from E. coli strain B was used. This was first purified by gel filtration on Sephadex G-100.

Preparation of Ribosomal Protein

The proteins associated with M. hominis and E. coli ribosomes were extracted using the LiCl-urea method of Spitnik-Elson (1965). An equal volume of 6 M LiCl in 8 M urea was added to a suspension of ribosomes (5-10 mg/ml) in 10 mM Tris·HCl (pH 7.8) containing 0.5 mM $Mg(C_2H_3O_2)_2$. After an incubation of 12-15 h at 4^o, the precipitated RNA was removed by centrifugation and the protein in the supernatant was dialyzed against 8 M urea to remove LiCl. Protein samples were treated at pH 8.1 with mercaptoethanol (0.06 M) for 3 h at 4^o to avoid artifacts due to aggregation resulting from disulfide bond formation (Hardy, et al., 1969). Recovery of protein was 95-98%, as estimated by the procedure of Lowry, et al., (1951) using bovine serum albumin as a standard.

Amino Acid Accepting Activity

Aminoacylation of tRNA was carried out in a reaction mixture containing, in a final volume of 0.1 ml (μmole unless otherwise specified): Tris·HCl (pH 7.8), 10; $Mg(C_2H_3O_2)_2$, 2.0; ATP (pH 7.0), 1.0; CTP, 0.01; KCl, 2.0; dithiothreitol, 0.1; bovine serum albumin, 10 μg; ^{14}C-L-amino acid (50 mCi/mmole), 0.005; tRNA, 15-150 μg; aminoacyl-tRNA ligases, 100 μg. The ligases, free of endogenous tRNA, were prepared as described by Muench and Berg (1966). After incubation at 37^o for 15 min, the mixtures were chilled and the reaction stopped by the addition of 7% trichloroacetic acid. The precipitates were collected on millipore filters, washed with 7% trichloroacetic acid, dried and counted.

Nucleotide Analysis of RNA

Two procedures were employed to determine the nucleotide composition of RNA. In the first, used to analyze for the major base components, the RNA was hydrolyzed at 37° with KOH (pH 13-14). After 25 h, the hydrolyzate was neutralized with $HClO_4$, the insoluble salt removed by centrifugation, and the clear supernatant used for analyses. Nucleotides were separated by paper chromatography (Whatman No. 1) as described by Lane (1963), eluted, and quantitated spectrophotometrically. The molar extinction coefficients of the nucleotides and nucleosides used in the base composition determinations are listed in Table 1. The minor base composition of RNA and nucleotide analysis of RNA samples available only in small amounts were determined by the more sensitive method developed by the Randeraths and their coworkers (E. Randerath, et al., 1968; K. Randerath, et al., 1969; K. Randerath and E. Randerath, 1969), which was, in the course of this investigation, extended to the quantitation of a number of modified nucleosides commonly found in RNA. This technique will be described in the Appendix.

Chromatographic and Analytical Techniques

Gel filtration was performed as described by Schleich and Goldstein (1966), using 1 M NaCl in 0.1% 1,1,1-trichloro-2-methyl-2-propanol hydrate (chlorotone), a bacteriostatic agent, to elute the RNA. Chromatography on MAK columns was carried out by the procedure of Mandell and Hershey (1960) as modified by Sueoka and Cheng (1967). The columns (0.9 x 35 cm) were equilibrated with 0.02 M Tris·HCl (pH 7.3) containing 0.3 M NaCl and the RNA was eluted with a linear NaCl gradient (0.3-1.0 M)

Table 1. Molar extinction coefficients used for determining nucleoside and nucleotide concentrations

Compound	pH	λ(nm)	E x 10^{-3}	Reference
A	6	260	14.9	Beaven et al., 1955
AMP	7	260	15.0	Ibid.
1MeA	1	259	13.1	Wacker and Ebert, 1959
6 MeA	7	266	15.9	Johnson et al., 1958
6,6-diMeA	7	275	18.8	Townsend et al., 1964
IPA	7	269	20.0	Robins et al., 1967
C	7	271	9.1	Beaven et al., 1955
CMP	7	260	7.6	Ibid.
5MeC	7	277	8.9	Fox et al., 1959
G	6	253	13.6	Beaven et al., 1955
GMP	7	260	11.4	Ibid.
1MeG	11	254	10.4	Broom et al., 1964
2MeG	11	254	14.8	Gerster and Robins, 1965
2,2-diMeG	13	262	14.3	K. Randerath, personal communication
7MeG	1	256	13.3	Jones and Robins, 1963
I	6	248.5	12.25	Beaven et al., 1955
U	7	262	10.1	Ibid.
UMP	7	260	10.0	Ibid.
H_2U	13	235	10.1	Janion and Shugar, 1960
Ψ	7	260	8.6	Cohn, 1960
3MeU	11.6	263	9.0	Thedford et al., 1965
rT	7	267	9.8	Fox et al., 1959
5FU	1	268	8.95	Yung et al., 1961

prepared in the same buffer.

Radioactive samples recovered from chromatography columns and sucrose gradients were precipitated with cold 7% trichloroacetic acid, filtered through glass fiber filters (Whatman GF/A), washed, dried, and counted. These filters as well as millipore filters used in assays of amino acid accepting activity were counted in a Packard liquid scintillation spectrometer in a mixture of toluene, 2,5-diphenyloxazole, and 1,4-bis-[2-(5-phenyloxazolyl)]-benzene.

Electrophoretic separation of ribosomal proteins on polyacrylamide gels (6 x 100 mm) was carried out at pH 4.5 by the procedure of Reisfeld, et al. (1962) with the modifications described by Leboy, et al. (1964). A current of 3 mA per gel was applied for 4 h. The gels were stained with 1% Amido Black in 7% acetic acid and destained electrophoretically in 7% acetic acid.

Analytical ultracentrifugations were performed at 3-4^{o} in a Spinco Model E ultracentrifuge equipped with both a photoelectric scanning system and schlieren optics. Sedimentation profiles obtained with the photoelectric scanner were recorded at 265 nm. Values for sedimentation coefficients were corrected to water at 20^{o}.

In determining the protein and RNA content of ribosomes, the ribonucleoprotein particles were extracted twice with hot 7% trichloroacetic acid. The acid insoluble material was dissolved in 0.1 N NaOH and its protein content estimated by the biuret reaction essentially as described by Gornall, et al. (1949) with bovine serum albumin as a standard. The amount of RNA in the acid soluble fraction was determined using the orcinol reaction (Dische, 1955) standardized

with E. coli tRNA.

Materials

The heart infusion broth, yeast extract, proteose peptones, and PPLO serum fraction used in culturing the mycoplasmas were all purchased from Difco Laboratories. [2-^{14}C]uracil and N,N-bis(2-hydroxyethyl)glycine (Bicine) are products of Calbiochem. [5-^{3}H]uridine (28.3 Ci/mM) was obtained from New England Nuclear Corporation. Worthington Biochemical Corporation was the source of supply for DNase (code DPFF), snake venom phosphodiesterase (code VPH), and bacterial alkaline phosphatase (code BAPF). Bovine pancreatic ribonuclease (Type 1-A) was purchased from the Sigma Chemical Co. [^{3}H]$NaBH_4$ (12.4 Ci/mM) was from the Amersham/Searle Corp. Cellulose thin-layer chromatography sheets (type 6064) were obtained from Eastman Organic Chemical Co. Acrylamide and N,N'-methylene-bisacrylamide, also from Eastman Organic Chemical Co., were recrystallized before use as described by Loening (1967). RNase free sucrose was purchased from Schwarz Bioresearch. Bovine Serum Albumin was Fraction V powder from the Pentex Corporation. Kieselguhr (Celite Hyflo Super Cel) was the product of the Johns-Mansville Co. Transfer RNA prepared from E. coli strain B was purchased from General Biochemicals and purified before use by gel filtration on Sephadex G-100. Nucleosides used as standards in the base composition analysis were obtained from the Cyclo Chemical Corporation. 1,1,1-Trichloro-2-methyl-2-propanol hydrate (chlorotone) was the product of the Aldrich Chemical Co. 2,5-Diphenyloxazole, 1,4-bis[2-(5-phenyloxazolyl)]-benzene, 2-(α-naphthyl)-5-phenyloxazole, and Cab-O-Sil gel powder were all purchased from the

Packard Instrument Company. Macaloid was obtained from the Baroid division of the National Lead Company. Royal Blue brand X-ray film was the product of the Eastman Kodak Company.

RESULTS

Properties of Mycoplasma Ribosomes

The sedimentation constants of ribosomes isolated from M. hominis were determined by extrapolating the $S_{20,w}$ values, measured at ribosome concentrations ranging from 0.06-3.2 mg/ml, to infinite dilution (Figure 1). Undissociated ribosomes were stable in buffers containing 1-5 mM Mg^{2+} or higher and had an $S^{o}_{20,w}$ of 71. In the presence of 0.1-0.5 mM Mg^{2+} they dissociated into subunits with sedimentation constants of 33S and 53S. These values are very similar to those observed for ribosomes from other prokaryotic cells. A more direct comparison of ribosomes from M. hominis and E. coli is shown in Figure 2, which depicts the results obtained when ^{3}H-labeled E. coli ribosomes were mixed with a 10 fold excess of mycoplasma ribosomes and centrifuged through a linear sucrose gradient in buffer containing 0.5 mM Mg^{2+}. It can be seen that the ribosomal subunits from the two species have identical sedimentation properties under these conditions.

Preliminary experiments with the mycoplasma ribosomes indicated they had different divalent cation requirements than did E. coli ribosomes. To explore this observation further, the effect of Mg^{2+} concentration on the dissociation and stability of mycoplasma ribosomes was examined. Aliquots of mycoplasma ribosomes (1.5 mg) in 10 mM Tris·HCl (pH 7.8) containing 0.5 mM Mg^{2+} were dialyzed against 2000-4000 volumes of the Tris·HCl buffer which contained $Mg(C_2H_3O_2)_2$ at concentrations ranging from 0.05-100 mM. The concentration of ribosomes in each sample was then adjusted to 2.5 mg/ml with the corresponding dialysis buffer, and $S_{20,w}$

Figure 1. Concentration dependence of the sedimentation coefficients of M. hominis ribosomes. Ribosomes (70S) were suspended in 10 mM Tris·HCl (pH 7.8) buffer containing 1.0 mM magnesium acetate; ribosomal subunits were in the same buffer containing 0.1 mM magnesium acetate. Centrifugation was carried out at 52,000 rev./min at 4°.

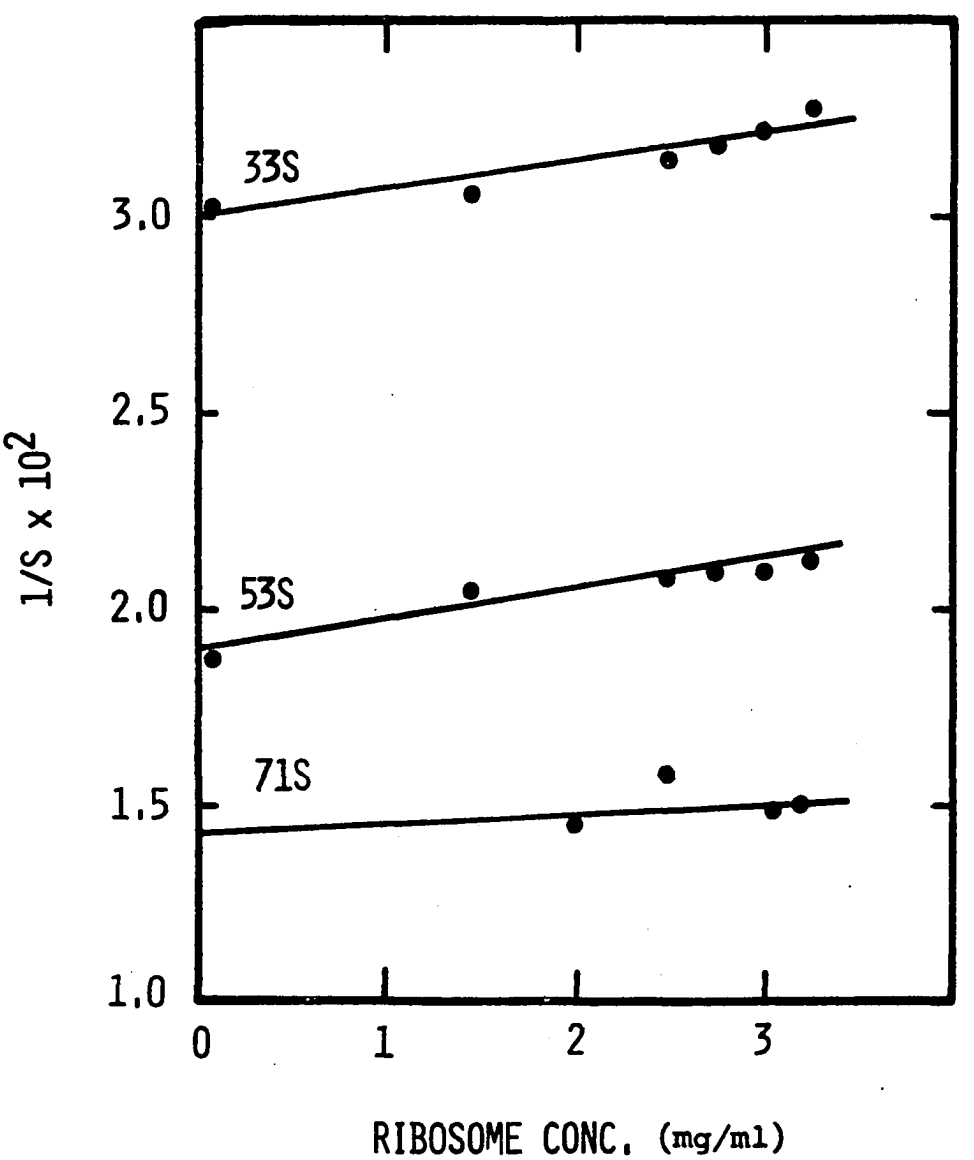
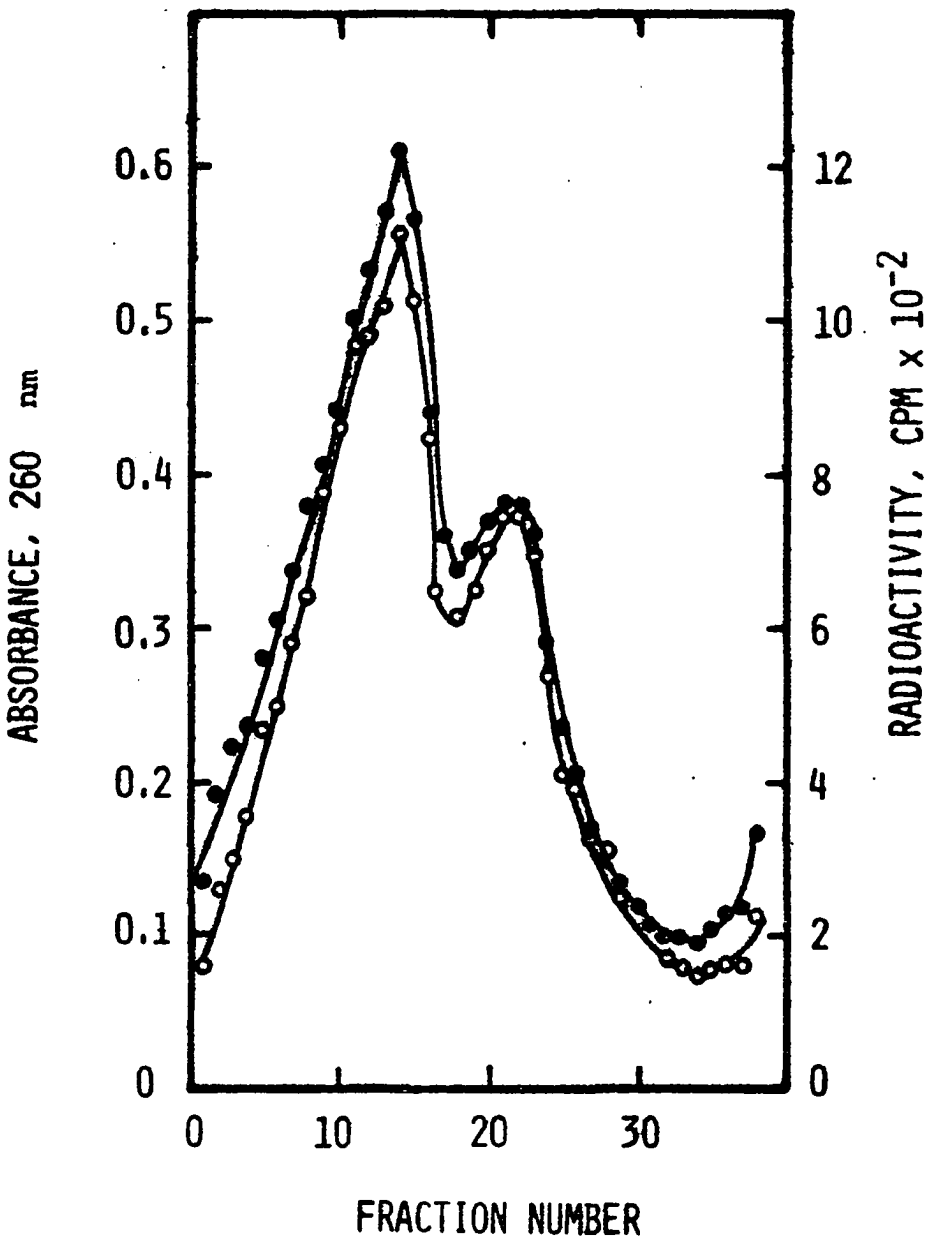

33S
53S
71S
3.0
2.5
2.0
1.5
1.0
1/S x 10²
0
1
2
3
RIBOSOME CONC. (mg/ml)

Figure 2. Sucrose density gradient centrifugation of ribosomes from M. hominis and E. coli. E. coli ribosomes isolated from cells grown in ^{3}H-uridine were mixed with a 10 fold excess of ribosomes prepared from M. hominis and the mixture was sedimented through a 5-20% linear sucrose gradient prepared in 10 mM Tris·HCl (pH 7.8) containing 0.5 mM magnesium acetate. Centrifugation was for 2 h at 39,000 rev/min in a Spinco SW39 rotor. (-●-●-), absorbance at 260 nm; (-o-o-), radioactivity.


ABSORBANCE, 260 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
RADIOACTIVITY, CPM x 10^{-2}
12
10
8
6
4
2
0
0
10
20
30
FRACTION NUMBER

values were determined using the schlieren optics of the analytical ultracentrifuge. Mass distribution among the various components observed in each sample was estimated by projecting an image of the schlieren pattern on drawing paper and measuring the area under individual peaks. Identical analyses of E. coli ribosomes were done to afford a standard for comparison. These results, presented in Table 2, show that there are indeed differences in the Mg^{2+} requirements of the M. hominis and E. coli ribosomes. These differences are most evident in the preparations which had been washed with 0.5 M NH_4Cl. Mycoplasma ribosomes required a Mg^{2+} concentration of 5 mM before any association of subunits was observable while similar preparations from E. coli showed a significant amount of association at 2 mM Mg^{2+}. At intermediate concentrations of Mg^{2+} (0.5 mM), all the ribosome preparations showed very similar behavior. This agrees well with the sucrose gradient analyses of mycoplasma and E. coli ribosomal subunits present in Figure 2. E. coli ribosomal subunits were stable at Mg^{2+} concentrations as low as 0.1 mM while at these levels of Mg^{2+} subunits of M. hominis ribosomes seemed to lose their structural integrity as evidenced by the appearance of peaks sedimenting at 17S and 23S and a concomitant loss of material sedimenting in the 30-45S range.

The protein and RNA composition of the ribosomes was determined and the data, presented in Table 3, show that ribosomes from M. hominis consist of 61% RNA and 39% protein. The virtually identical results obtained for E. coli ribosomes again indicates the similarity of mycoplasma ribosomes to those of eubacteria. Washing the M. hominis ribosomes with high salt concentrations (0.5 M NH_4Cl) had no significant effect on the

Table 2. The effect of Mg^{2+} concentration on M. homonis ribosomes. Ribosomes prepared as described in Experimental, both with and without a 0.5 M NH_4Cl wash, were dialyzed against 2000-4000 volumes of 10 mM Tris·HCl buffer (pH 7.8) containing Mg^{2+} at the concentration indicated. Following dialysis, the concentration of ribosomes in each sample was adjusted to 2.5 mg/ml and $S_{20,w}$ values determined in the analytical ultracentrifuge

Ribosomes	0.5 M NH_4Cl Wash	mM $Mg(C_2H_3O_2)_2$	$S_{20,w}$	Mass Distribution
M. hominis	-	100	5, 27, 57, 68	not computed *
"	-	10	32, 49, 65	not computed *
"	-	1	31, 47, 60	0.27,0.66,0.07
"	-	0.5	30, 45	0.34,0.66
"	-	0.1	29, 44	0.36,0.64
"	-	0.05	17, 24, 44	0.82,0.18 **
M. hominis	+	100	3, 25, 56, 68	not computed *
"	+	10	30, 48, 60	not computed *
"	+	5	30, 46, 63	0.21,0.79,trace
"	+	1	30, 45	0.27,0.73
"	+	0.7	30, 46	0.26,0.74
"	+	0.5	30, 46	0.28,0.72
"	+	0.5	29, 44	0.30,0.70
"	+	0.2	30, 44	0.30,0.70
"	+	0.1	17, 23, 44	0.11,0.35,0.53
"	+	0.07	17, 23, 45	0.59,0.41**
"	+	0.05	17, 23, 38	0.90,0.10**
E. coli	+	2	30, 46, 61	0.17,0.67,0.16
"	+	0.7	30, 46	0.29,0.71
"	+	0.5	29, 46	0.33,0.66
"	+	0.2	29, 44	0.32,0.68
"	+	0.1	29, 44	0.30,0.70
"	+	0.07	28, 35, 42	0.37,0.19,0.44

*Aggregation at high Mg^{2+} concentrations resulted in poor resolution of the peaks, therefore accurate determinations for individual components were not possible.

**The first number is the sum of the two slower components; the resolution being insufficient to allow separate determinations.

Table 3. The RNA and protein content of *M. hominis* and *E. coli* ribosomes. Details are described in Experimental. Values reported are the averages of three determinations

Ribosomes	0.5 M NH_4Cl Wash	mg Protein**	mg RNA*	% RNA
M. hominis	-	6.3	9.7	61
M. hominis	+	4.5	6.9	61
E. coli	+	4.5	6.4	59

* Determined by the orcinol procedure described by Dische (1955).

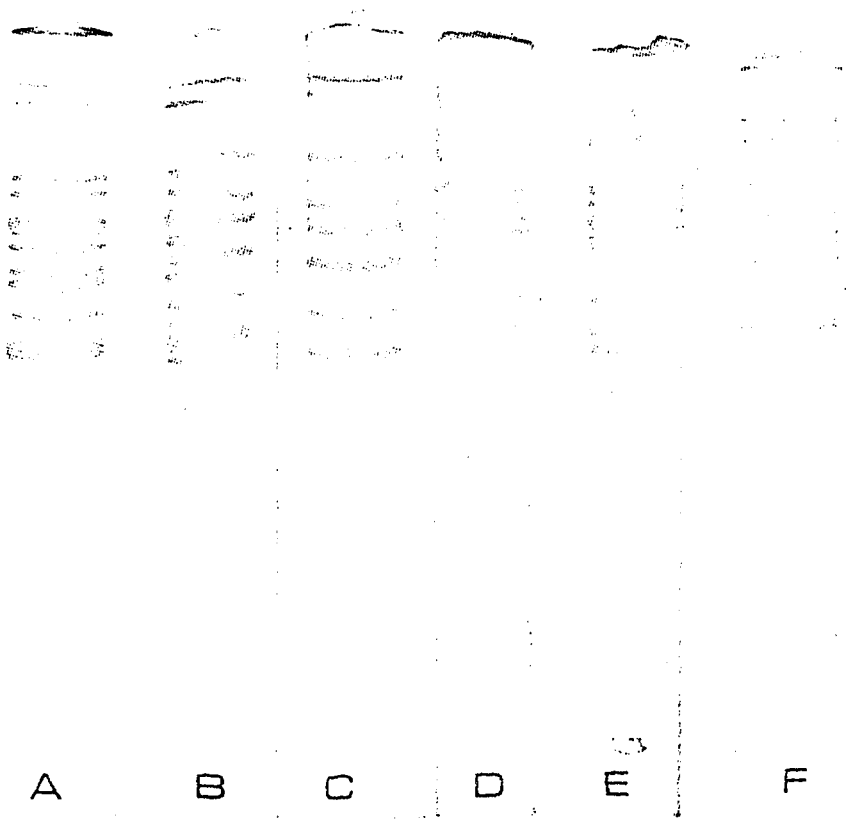
**Measured by the biuret procedure of Gornall *et al.*, (1949).

RNA: protein ratio. These observations are in contrast to those of Kirk and Morowitz (1969) who found that ribosomes from M. gallisepticum prepared with low-salt washes had a low RNA: protein ratio and that substantial amounts of protein were removed when they were washed in buffer containing 2 M KCl.

Ribosomal Proteins

Proteins isolated from M. hominis ribosomes were examined by polyacrylamide gel electrophoresis at pH 4.5 to determine whether ribosomes from a genetically limited organism such as mycoplasma contain significantly fewer protein components than those of more complex organisms. The gel patterns obtained are shown in Figure 3. Eighteen bands were resolved electrophoretically from the protein of the 30S subunit and 21 from the proteins of the 50S subunit. For comparison, the protein complement of E. coli ribosomes was also examined (Figure 3). E. coli 30S and 50S subunits contained respectively, 15 and 20 distinct protein components. The more acidic ribosomal proteins from these two organisms were compared by examining the gel patterns obtained when the electrophoresis was carried out at pH 8.7 with migration being toward the anode. Under these conditions, 7-8 components were observed in preparations derived from NH_4Cl washed 70S ribosomes of both M. hominis and E. coli (results not shown). Hence, the mixture of proteins present in mycoplasma ribosomes is at least as complex as that in the ribosomes of more extensively investigated organisms. When electrophoretic mobilities of the ribosomal proteins from the two organisms were compared on split gels, the results, also seen in Figure 3, indicate that about 50% of the

Figure 3. Polyacrylamide gel electrophoresis of ribosomal proteins. (A) M. hominis 30S proteins. (B) Split gel comparing the proteins from the 30S subunits of M. hominis and E. coli. (C) E. coli 30S proteins. (D) M. hominis 50S proteins. (E) Comparison of M. hominis and E. coli 50S proteins. (F) E. coli 50S proteins.


A
B
C
D
E
F

mycoplasma ribosomal proteins do not correspond to any found in E. coli. Three additional bands were observed near the origin in gel patterns from the 70S ribosomes of M. hominis when the proteins were not reduced with 2-mercaptoethanol before electrophoresis indicating a tendency of these proteins to aggregate through disulfide bond formation.

Isolation and Characterization of M. hominis RNA

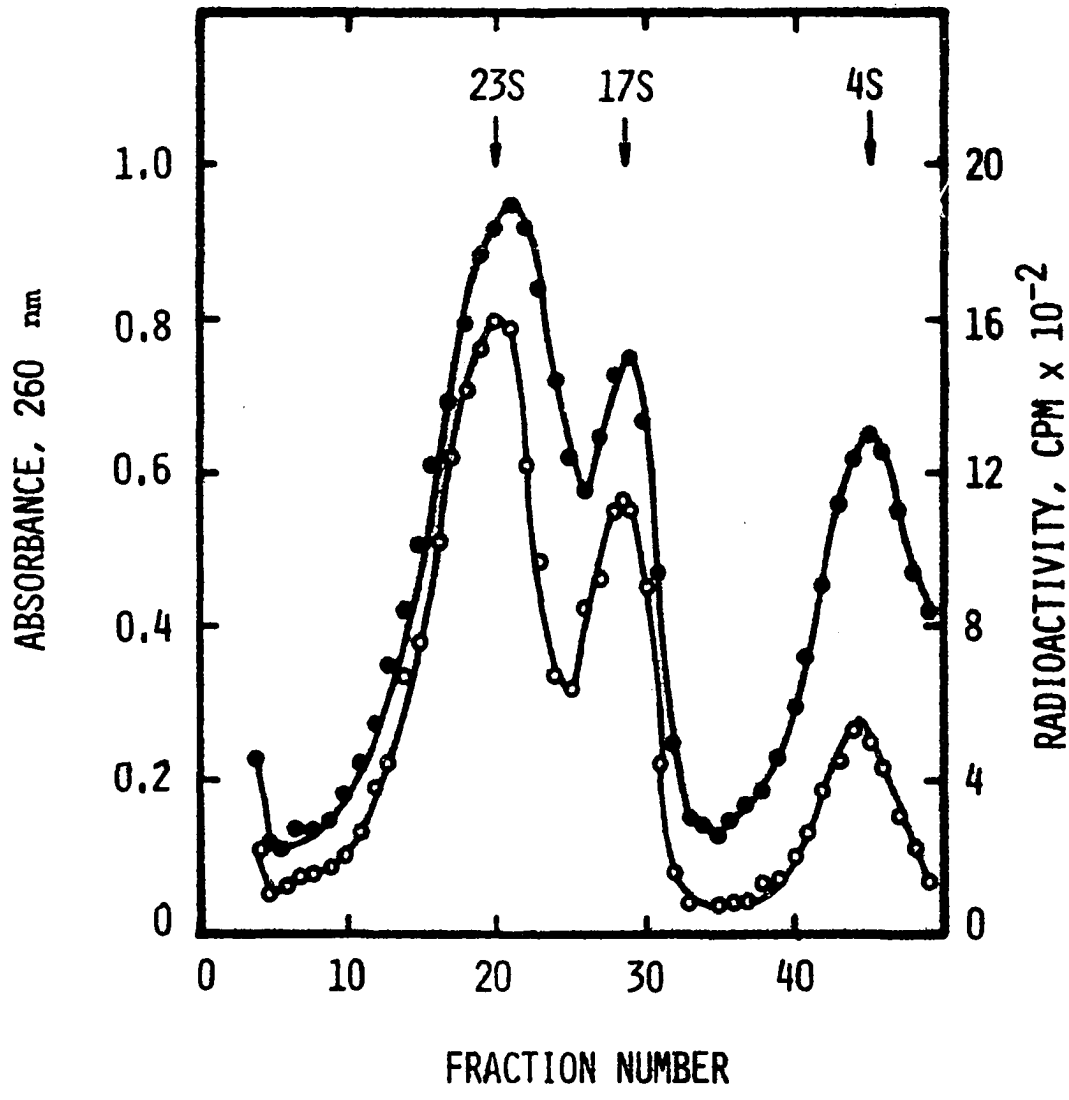
M. hominis is known to possess a high level of endogenous ribonuclease activity with 85-90% of the enzyme associated with the cell membrane (Pollack, et al., 1965). This may account for some of the difficulties encountered in the preparation of undegraded high molecular weight RNA from this organism. Attempts to extract rRNA from purified ribosomes using SDS (Kurland, 1960), SDS and phenol (Kurland, 1960), or SDS and a chloroform-pentanol mixture (Ingle and Burns, 1968) either with or without bentonite (Fraenkel-Conrat, et al., 1961) or Macaloid (Stanley and Bock, 1965) as ribonuclease inhibitors all failed to yield reproducible preparations of RNA. RNA isolated by digestion of mycoplasma ribosomes with pronase either in the presence or absence of diethyl pyrocarbonate, a ribonuclease inhibitor (Solymosy, et al., 1968), also appeared to be degraded. When RNA prepared by any of the methods just enumerated was examined in the analytical ultracentrifuge, the schlieren patterns observed varied considerably with none of the techniques giving reproducible results. In many preparations no material sedimenting faster than 4S was observed. When high molecular weight material was present, there were generally two components with sedimentation coefficients of 11S-16S and 18S-22S. The amount of the 11S-16S component

was always greater than that of the 18S-22S; an unusual result if compared with the patterns observed for rRNA from virtually all other organisms.

Attempts to extract intact RNA from whole cells as done by Kirk and Morowitz (1969) with M. gallisepticum also resulted in preparations which appeared to be degraded when examined on sucrose density gradients and in the analytical ultracentrifuge. This may be due to the greater amount of endogenous RNase activity in M. hominis (Pollack, et al., 1965). However, since the bulk of this enzyme is associated with the cell membrane, intact RNA could be prepared from crude cell extracts, as described in Experimental, if the membrane fraction was removed by centrifugation immediately after the cells had been lysed.

The sedimentation properties of RNA prepared in this manner were compared with those of E. coli rRNA by mixing ^{3}H-labeled E. coli RNA with a 10 fold excess of M. hominis RNA and centrifuging the mixture in a sucrose density gradient (Figure 4). Sedimentation coefficients of 22S, 17S, and 4S can be calculated for the constituents present in M. hominis RNA using the method of Martin and Ames (1961) and assuming the larger species of E. coli RNA sediments at 23S. $S_{20,w}$ values for the two high molecular weight components of M. hominis RNA were determined in the analytical ultracentrifuge and the values obtained, 23S and 16S, agree well with those measured by sucrose density gradient centrifugation. Figure 4 shows that the peaks of 16-17S RNA from both species coincided while the larger RNA component from E. coli sedimented slightly faster than that from M. hominis. To test the possibility that this difference resulted from degradation of mycoplasma RNA during the extraction

Figure 4. Sucrose density gradient centrifugation of RNA from M. hominis and E. coli. E. coli RNA, prepared from cells grown in [^{14}C]uracil was mixed with a 10 fold excess of M. hominis RNA and centrifuged through a 5-20% linear sucrose gradient which contained 0.02 M sodium acetate (pH 5.5) and 0.15 M KCl. Centrifugation was for 15 h at 22,000 rev./min in a Spinco SW25.1 rotor (-●-●-), absorbance at 260 nm; (-o-o-), radioactivity.


23S
17S
4S
1.0
0.8
0.6
0.4
0.2
0
20
16
12
8
4
0
0
10
20
30
40
ABSORBANCE, 260 nm
RADIOACTIVITY, CPM x 10^{-2}
FRACTION NUMBER

procedure, E. coli RNA labeled with [^{14}C]uracil was mixed with a mycoplasma cell lysate and RNA prepared from the mixture by phenol extraction as described in Experimental. This RNA was then analyzed by sucrose gradient centrifugation and the results were essentially identical to those shown in Figure 4. No degradation of the E. coli RNA was observable and the small difference in sedimentation rate between the larger rRNA component of E. coli and M. hominis was still evident. It thus seems unlikely that RNase activity can account for the difference observed. It can also be seen in Figure 4 that the low molecular weight RNA from mycoplasma sediments at the same rate (4S) as that from E. coli.

Total RNA from M. hominis, prepared so as to minimize degradation, was separated into its constituents by gel filtration on a 4 cm x 40 cm column of Sephadex G-100 (Figure 5). Three fractions were effectively resolved by this procedure: high molecular weight rRNA, 5S RNA, and tRNA, each of which was then further characterized.

The ribosomal RNA fraction, which eluted in the excluded volume of the Sephadex column (fractions 11-17 in Figure 5) consisted of the two components described earlier. As mentioned previously, these had $S^{o}_{20,w}$ values of 16S and 23S as determined in the analytical ultracentrifuge in buffer containing 0.02 M $NaC_2H_3O_2$ (pH 5.5) and 0.15 M KCl. The mass ratio of 23S:16S found in this fraction varied from 1.5-2.0.

The M. hominis RNA peak eluting from Sephadex G-100 just after the high molecular weight RNA (fractions 24-28 in Figure 5) contained 5S RNA which was identified by comparison with 5S RNA from E. coli. The two behaved identically on Sephadex G-100 (Figure 6); a mixture of ^{14}C-labeled

Figure 5. Gel filtration on Sephadex G-100 of M. hominis RNA. RNA was prepared from crude cell extracts of M. hominis as described in Experimental. The sample was applied to a 4 cm x 40 cm column of Sephadex G-100 and eluted with 1.0 M NaCl. Fractions pooled for further analysis were: rRNA, 14-17; 5S RNA, 24-28; tRNA, 30-34.

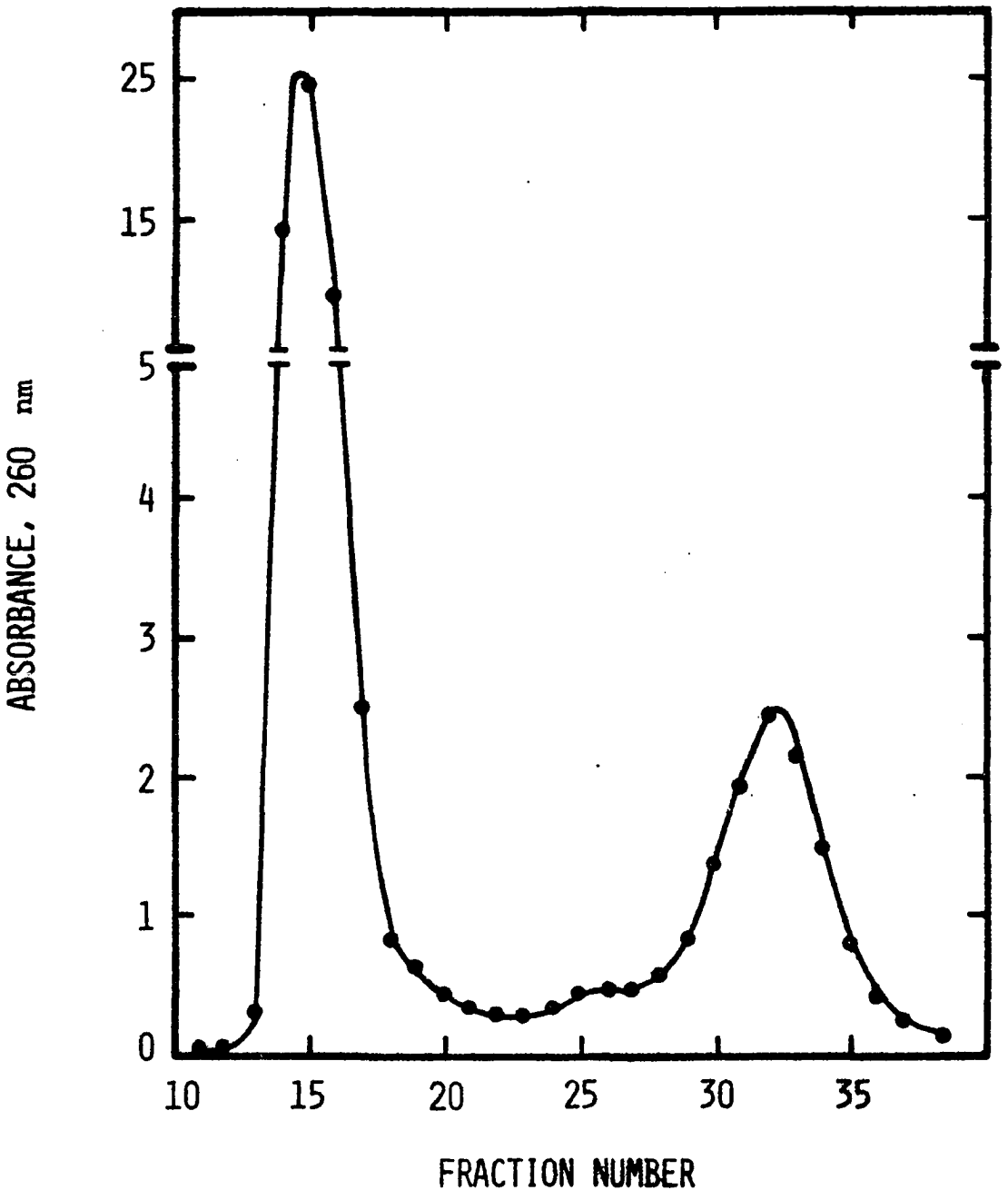
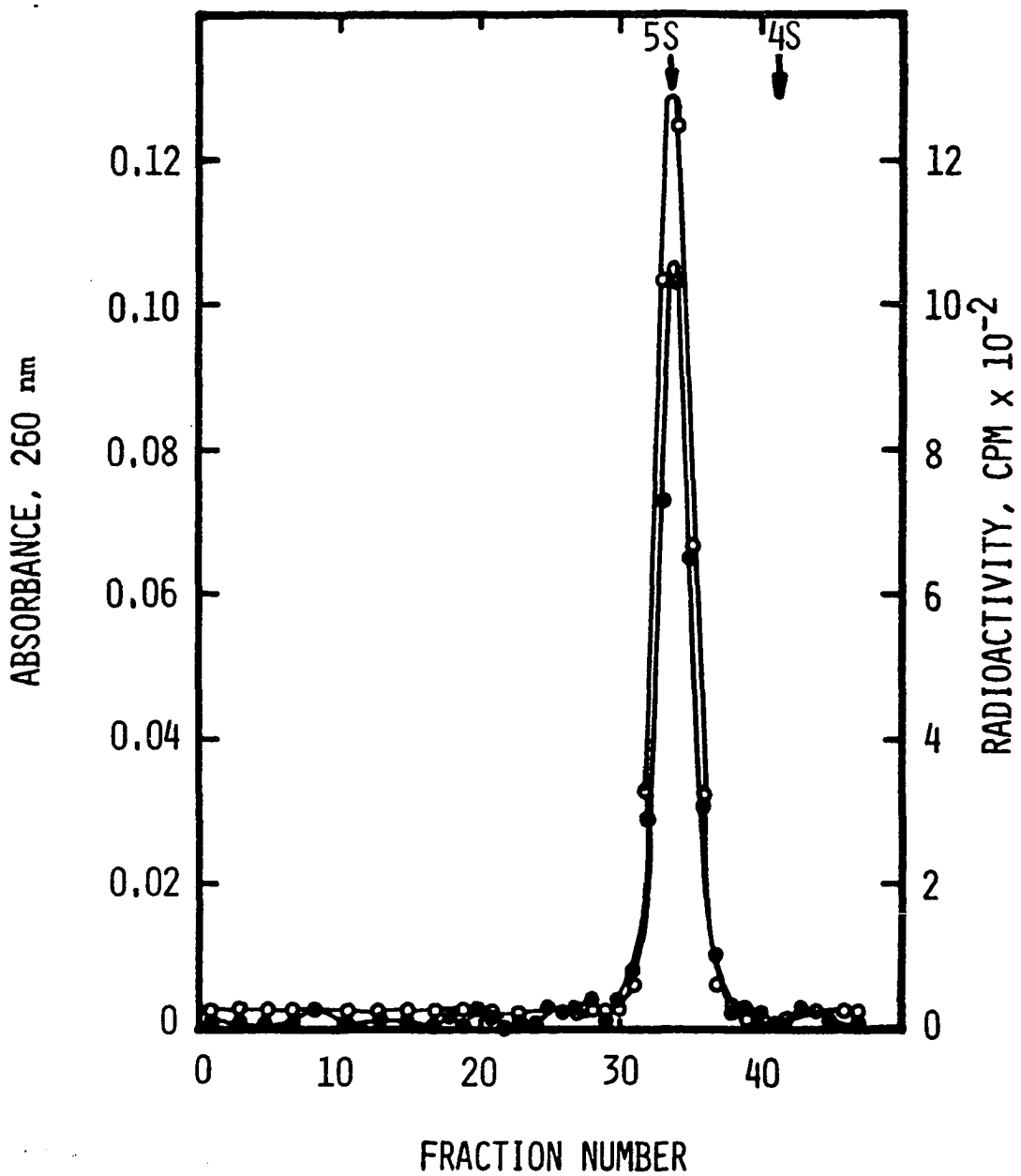

ABSORBANCE, 260 nm
25
15
5
4
3
2
1
0
10
15
20
25
30
35
FRACTION NUMBER

Figure 6. Gel filtration on Sephadex G-100 of mycoplasma and E. coli 5S RNA. M. hominis 5S RNA (from fractions 24-28 of Fig. 5), recovered by ethanol precipitation and purified by rechromatography on Sephadex G-100, was mixed in 15 fold excess with ^{14}C-labeled E. coli 5S RNA, which had also been purified by gel filtration. This mixture was applied to a 1.5 cm x 150 cm column of Sephadex G-100 and eluted with 1.0 M NaCl. Fractions of 4.5 ml were collected. Arrows indicate the positions at which E. coli tRNA and 5S RNA are known to elute. (-●-●-), absorbance at 260 nm; (-o-o-), radioactivity.

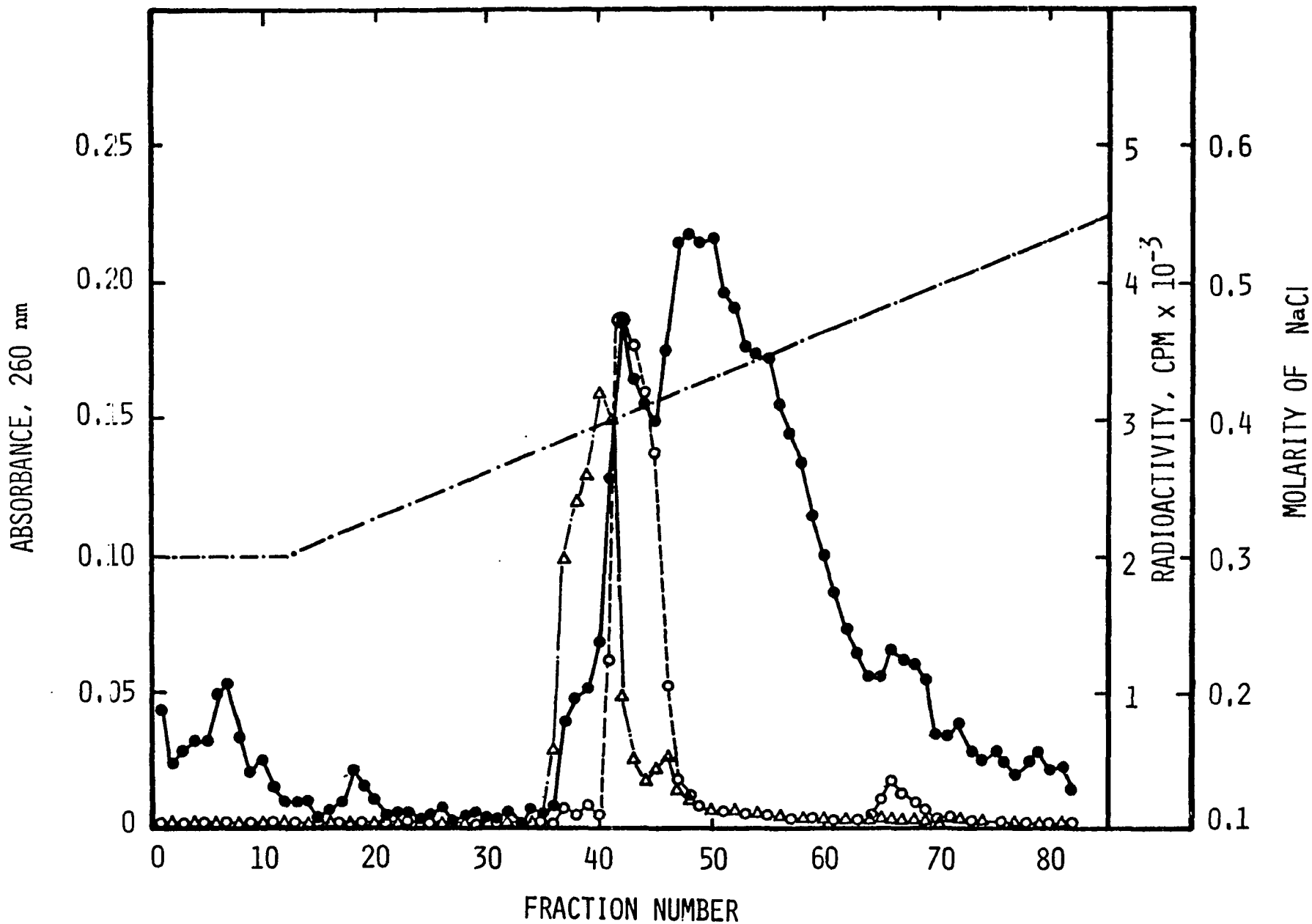

5S
4S
0.12
0.10
0.08
0.06
0.04
0.02
0
12
10
8
6
4
2
0
0
10
20
30
40
ABSORBANCE, 260 nm
RADIOACTIVITY, CPM x 10^{-2}
FRACTION NUMBER

E. coli 5S RNA and the mycoplasma RNA recovered from fractions 24-28 of Figure 5 chromatographed as one peak.

Chromatography on MAK columns revealed differences between mycoplasma and E. coli 5S RNA (and tRNA) (Figure 7). M. hominis 5S RNA (0.9 mg) purified by a second round of gel filtration on Sephadex G-100 was mixed with ^{14}C-labeled tRNA (0.13 mg) and ^{3}H-labeled 5S RNA (0.2 mg), both from E. coli, and the mixture separated on a 0.9 cm x 35 cm column of MAK as described in Experimental. The results show that mycoplasma 5S RNA, the major peak of UV absorbing material in Figure 7, is eluted from MAK at a higher salt concentration than either E. coli 5S RNA or tRNA. Absorbance at 260 nm coinciding with the radioactive peaks is contributed by the labeled E. coli 5S and tRNA. The specific activity of the labeled RNA species was low and relatively large amounts were mixed with the M. hominis 5S RNA; they contributed 25% of the total UV absorbing material put on the column.

A calculation of the amount of 5S RNA present in the mycoplasma ribosomes was made by assuming that the molecular weights of 5S, 16S, and 23S RNA from M. hominis were equivalent to those of the corresponding molecules from E. coli; $4 \cdot 10^4$, $0.55 \cdot 10^6$, and $1.1 \cdot 10^6$, respectively (Spirin and Gavrilova, 1969). The total UV absorbance in fractions 11-17 of Figure 5 (460 A_{260} units) represents the 16S + 23S RNA. Fractions 24-28 of Figure 4 were rechromatographed on a 1.5 cm x 150 cm column of Sephadex G-100 and found to contain 10.0 A_{260} units of 5S RNA. From this data a molar ratio of 0.9:1 for 5S:16S + 23S RNA was calculated. This ratio is in good agreement with the general observation of 1 mole

Figure 7. Chromatography on MAK columns of 5S RNA from M. hominis. M. hominis 5S RNA (0.9 mg) was mixed with ^{14}C-labeled tRNA (0.13 mg; $1.7 \cdot 10^5$ cpm/mg) and ^{3}H-labeled 5S RNA (0.2 mg; $1.2 \cdot 10^5$ cpm/mg) both from E. coli. This mixture was applied to a 0.9 cm x 35 cm column of MAK and the RNA eluted with a linear gradient of NaCl in 10 mM Tris·HCl buffer (pH 7.3). Fractions of 5 ml were collected. All RNA species used for chromatography on MAK columns were first purified to apparent homogeneity by gel filtration on Sephadex G-100. (-●-●-), absorbance at 260 nm; (-o-o-), ^{3}H-radioactivity; (-Δ-Δ-), ^{14}C-radioactivity.


ABSORBANCE, 260 nm
0.25
0.20
0.15
0.10
0.05
0
RADIOACTIVITY, CPM x 10^{-3}
5
4
3
2
1
MOLARITY OF NaCl
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

of 5S RNA per 50S ribosome (Rosset, et al., 1964).

The third peak of mycoplasma RNA resolved on Sephadex G-100 (fractions 30-34 of Figure 5) contained transfer RNA. Gel filtration and sedimentation studies indicate this RNA is approximately the same size as E. coli tRNA (Figure 8). Chromatography, on Sephadex G-100, of a mixture containing an excess of M. hominis tRNA and ^{14}C-labeled E. coli tRNA gave the results shown in Figure 8a; the elution pattern of the mycoplasma tRNA (A_{260} profile) coincides with that of the ^{14}C-labeled E. coli tRNA. Examination of a similar mixture of M. hominis tRNA with ^{3}H-labeled E. coli tRNA on a 5-20% sucrose gradient shows that the two RNAs have essentially identical sedimentation coefficients (Figure 8b).

The tRNA from M. hominis could be charged by aminoacyl-tRNA ligases from E. coli. Comparison of E. coli tRNA and that from M. hominis showed that E. coli tRNA had a somewhat greater capacity for accepting phenylalanine and proline than mycoplasma tRNA, while the reverse was true for isoleucine (Figure 9). It is not known whether these differences are due to inherent differences in the ability of these tRNAs to function as substrates for the E. coli enzymes or to differences in the relative abundance of specific tRNA species in these preparations.

Nucleotide Composition of M. hominis RNA

The nucleotide composition of each of the three fractions of M. hominis RNA separated by gel filtration was determined either spectrophotometrically, when mg quantities of RNA were available, or by the more sensitive method developed by Randerath and his coworkers (1969) (see Appendix for details). This latter procedure was especially useful for

Figure 8. Gel filtration and sedimentation properties of tRNA from M. hominis. tRNA (recovered from fractions 30-34 of Fig. 5) was mixed in 15 fold excess with either (a) ^{14}C-labeled E. coli tRNA and chromatographed on a 1.5 cm x 150 cm column of Sephadex G-100 (fraction volume, 5.5 ml) or (b) ^{3}H-labeled E. coli tRNA for centrifugation through a linear 5-20% sucrose gradient prepared in 0.02 M sodium acetate buffer (pH 5.5) containing 0.15 M KCl. Centrifugation was for 24 h at 39,000 rev/min in a Spinco SW39 rotor. (-●-●-), absorbance at 260 nm; (-o-o-), radioactivity.

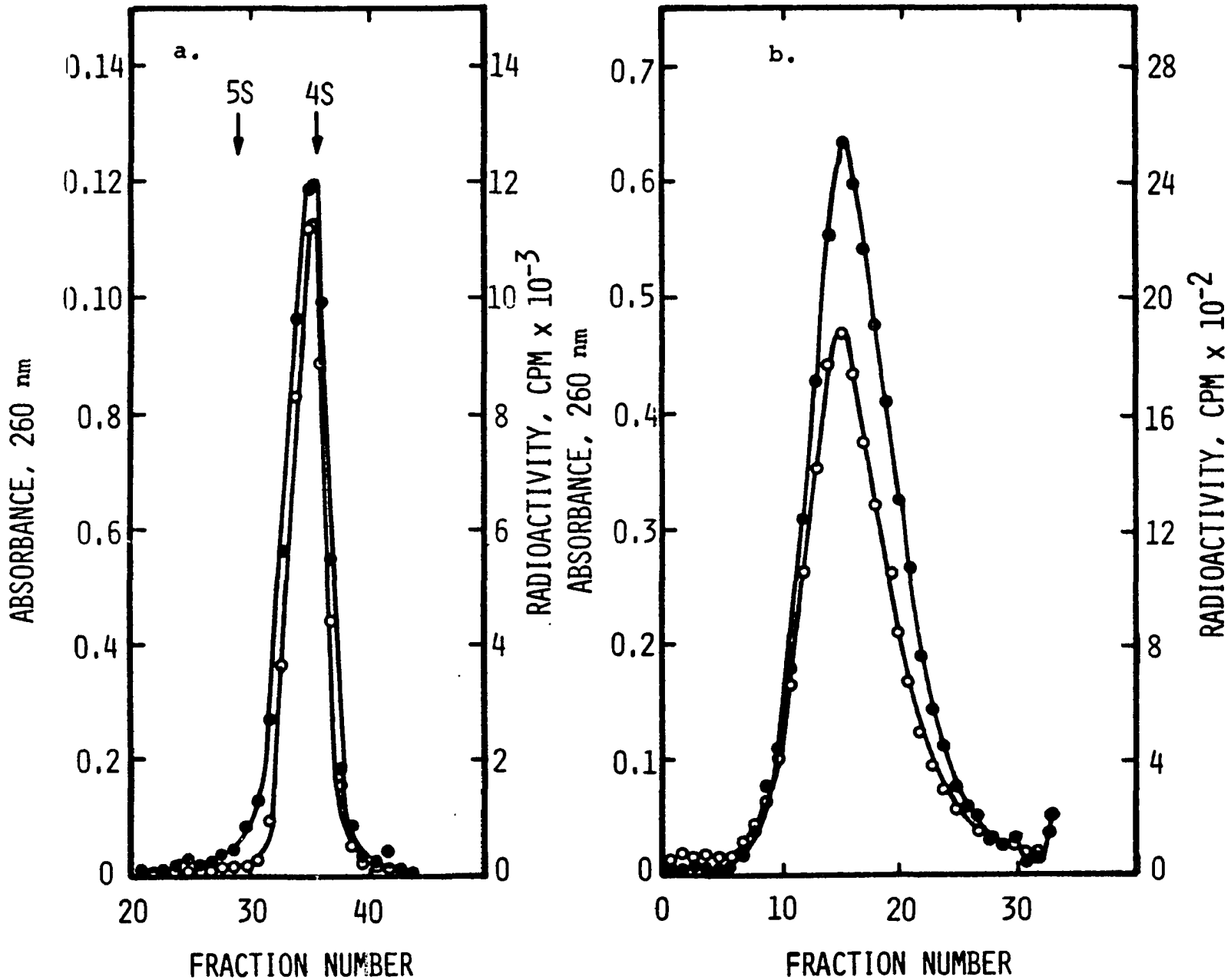
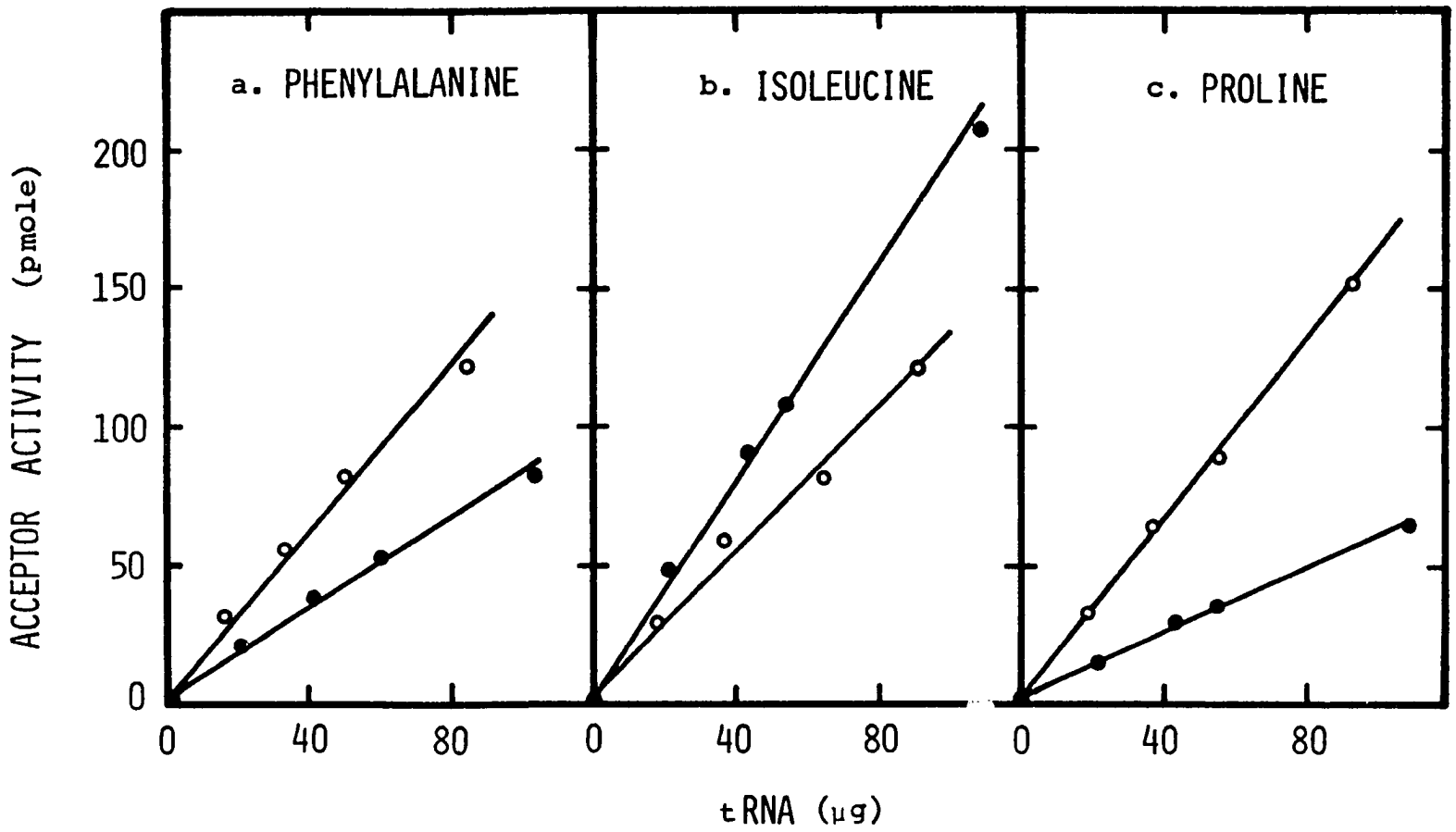

a.
5S
4S
ABSORBANCE, 260 nm
RADIOACTIVITY, CPM x 10^{-3}
FRACTION NUMBER
b.
ABSORBANCE, 260 nm
RADIOACTIVITY, CPM x 10^{-2}
FRACTION NUMBER

Figure 9. Amino acid accepting activity of tRNA from M. hominis. M. hominis tRNA (from fractions 30-34 of Fig. 5) and E. coli tRNA were assayed for their capacity to accept phenylalanine (a), isoleucine (b), and proline (c) as described in Experimental. The values reported have been corrected for the amount of amino acid incorporated in controls containing no added tRNA (2-10 pmoles). (-●-●-) M. hominis tRNA; (-o-o-), E. coli tRNA.


a. PHENYLALANINE
b. ISOLEUCINE
c. PROLINE
ACCEPTOR ACTIVITY (pmole)
200
150
100
50
0
0
40
80
tRNA (µg)

quantitating the modified nucleotide constituents and analyzing the base composition of samples difficult to obtain in large quantities. The data for the major nucleotides, presented in Table 4, shows that all three species of mycoplasma RNA have a lower G + C content than the corresponding E. coli RNA. This difference is greatest in 5S RNA, where the mole % G + C is 43.1% for the RNA from M. hominis compared to 63.7% for that from E. coli.

Several other differences between M. hominis and E. coli tRNA were revealed by comparing the modified nucleotide content of the transfer RNAs from the two organisms (Table 5). The most striking difference is the virtual absence of rT in the tRNA from M. hominis. In addition, the levels of a number of other minor constituents are considerably lower in mycoplasma tRNA. The Ψ and H_2U content is 40-50% lower when compared to E. coli tRNA. There also appears to be less 4TU. It is not possible to determine this constituent by the method of Randerath and collaborators (1969) since it is oxidized by $NaIO_4$ (Ziff and Fresco, 1968) and subsequently coverted to uridine. The presence of 4TU in M. hominis tRNA was determined spectrophotometrically as described by Lipsett (1966). The absorbance maximum for 4TU at neutral pH, 336 nm, is far enough above that of any other known RNA constituent to allow a direct spectrophotometric estimation of this nucleoside. A characteristic shift in absorbance maxima from 336 nm to 311 nm when the pH is increased from 7.0 to 12 provides an additional criterion for the identification of 4TU in RNA. The spectra of both E. coli and M. hominis tRNA showed prominent absorption peaks at 336 nm at pH 7.0 which shifted to 311 nm

Table 4. Nucleotide composition of RNA from M. hominis and E. coli. The procedures used for base composition analysis are detailed under Experimental, (Method I) and in the Appendix (Method II). Reference to these techniques in the table is as follows: Method I-Spectrophotometric quantitation of nucleotides separated by paper chromatograph. Method II-Radioactivity measurements of ^{3}H-labeled nucleoside trialcohols separated by thin-layer chromatography

Type	Source	Method of Analysis	Nucleotide constituent (Mole %) G	C	A	U	G+C
rRNA	M. hominis	I	26.9	19.5	28.8	24.7	46.4
	E. coli	I	32.1	21.9	25.5	20.5	54.0
5S	M. hominis	II	22.3	20.8	28.8	28.1	43.1
	E. coli	*	33.7	30.0	19.2	17.1	63.7
tRNA	M. hominis	I	29.5	26.6	21.3	22.7	56.1
	M. hominis	II	29.3	27.1	21.1	22.4**	56.4
	E. coli	I	31.5	30.1	19.9	18.5	61.6
	E. coli	II	30.0	30.4	20.6	19.0**	60.4

*Calculated from the known sequence of E. coli 5S RNA (Brownlee et al., 1968).

**Represents the sum of uridylate, pseudouridylate, and ribothymidylate which are resolved by thin-layer chromatography, but not on paper.

Table 5. Modified nucleoside content of M. hominis tRNA. The amounts of a number of modified nucleosides present in the tRNA of M. hominis were determined by the method of Randerath et al., (1969) as described in the Appendix. For comparison, results of analyses done on E. coli tRNA are also presented. The number of determinations for each value is given in parentheses

Nucleoside	Mole %				Residues per tRNA Molecule*	
	M. hominis		E. coli		M. hominis	E. coli
Ψ	1.28±0.28	(11)	2.24±0.08	(6)	1.02	1.79
H_2U	1.18±0.26	(11)	1.95±0.12	(6)	0.94	1.56
rT	<0.01	(11)	1.24±0.08	(5)	<0.01	0.99
3MeU	0.06±0.05	(5)	<0.01	(2)	0.05	<0.01
5MeC	0.03±0.02	(7)	0.01	(2)	0.02	<0.01
I	0.15±0.03	(11)	0.17±0.03	(4)	0.12	0.14
1MeA**	0.27±0.15	(11)	0.08±0.03	(2)	0.22	0.06
6MeA**	0.44±0.12	(11)	<0.01	(2)	0.35	<0.01
6,6-diMeA	0.05±0.01	(7)	<0.01	(2)	0.04	<0.01
2MeG	0.03±0.01	(7)	0.04±0.01	(2)	0.02	0.03
7MeG***	0.80±0.10	(7)	0.40±0.05	(3)	0.64	0.32

*Calculated assuming 80 nucleotide residues per tRNA molecule.

**Corrected for the conversion of one third of the 1MeA to 6MeA.

***Corrected for 80% loss during analysis (see Appendix for details).

when the pH was raised to 12 (Table 6). This change was identical in both wavelength and intensity in the two types of RNA. An estimate of the relative amounts of 4TU present in the tRNA from E. coli and M. hominis can be made by comparing the $A_{336}:A_{260}$ ratio (pH 7.0) of the two samples. This ratio was found to be $1.7 \cdot 10^{-2}$ and $0.77 \cdot 10^{-2}$ for tRNA from E. coli and M. hominis respectively (Table 6) thus, the 4TU content of the mycoplasma tRNA is less than one-half that of E. coli. If the latter is presumed to contain 0.8 mole % 4TU (Lipsett, 1966), the 4TU content of M. hominis tRNA is approximately 0.4 mole %.

Nucleoside analyses of separated 16S and 23S ribosomal RNA components are shown in Table 7; comparison of the rRNA from the 2 species shows the levels of modified pyrimidines in M. hominis to be equal to or less than those in the corresponding RNA from E. coli. Both rRNA components from mycoplasma, as well as the 16S RNA of E. coli lack rT. The H_2U content of all four species of rRNA is, within standard deviations, equivalent. The Ψ content of 23S RNA is lower in M. hominis than in E. coli while the 16S RNA from both organisms contains about the same amount of this nucleoside. The distribution of minor purine components in the rRNA from the two organisms shows no clear patterns or trends.

Table 6. Spectrophotometric determination of the 4TU content of *M. hominis* tRNA. Absorbance measurements of *M. hominis* and *E. coli* tRNA were taken at pH 7.0 (0.05 M phosphate) and pH 12.0 (0.01 M KOH) in a Cary Model 15 recording spectrophotometer

	Absorbance Ratios (%)				
	M. hominis		*E. coli*		*M. hominis* / *E. coli*
	$\frac{A_{336}}{A_{260}}$	$\frac{A_{311}}{A_{260}}$	$\frac{A_{336}}{A_{260}}$	$\frac{A_{311}}{A_{260}}$	$\frac{A_{336}}{A_{260}}$
pH 7.0	0.77	0.85	1.70	1.45	45.3
pH 12.0	0.51	1.27	1.14	2.20	44.7
$\frac{\text{pH } 7.0}{\text{pH } 12.0}$	151	66.9	149	65.9	-----

Table 7. Nucleotide composition of *M. hominis* ribosomal RNA. The two species of high molecular weight RNA from *M. hominis* were isolated by sucrose density gradient centrifugation and the nucleotide composition of each form was determined using the procedure described in the Appendix. The results from similar analyses of 16S and 23S RNA from *E. coli* are presented for comparison. Each value is the average of three determinations

Nucleoside	Mole %			
	23S RNA		16S RNA	
	M. hominis	*E. coli*	*M. hominis*	*E. coli*
G	25.4±0.1	29.1±0.9	26.0±0.1	28.7±0.7
C	19.3±0.2	22.4±0.3	19.5±0.5	23.5±0.2
A	29.5±0.2	26.8±0.4	29.5±0.4	25.7±0.6
U	25.2±0.1	20.8±0.8	23.9±0.6	21.3±0.2
Ψ	0.20±0.2	0.34±0.05	0.13±0.03	0.19±0.02
H_2U	0.09±0.03	0.10±0.02	0.08±0.02	0.07±0.01
rT	<0.01	0.08±0.01	<0.01	<0.01
3MeU	0.02±0.01	0.02±0.01	0.04±0.01	0.05±0.01
5MeC	<0.01	<0.01	0.15±0.02	0.18±0.03
I	0.09±0.01	0.19±0.01	0.16±0.04	0.03±0.02
1MeA	<0.01	<0.01	<0.01	<0.01
6MeA	0.01±0.01	0.07±0.01	<0.01	<0.01
6,6-diMeA	<0.01	0.01±0.01	<0.01	<0.01
2MeG	0.04±0.01	0.07±0.01	0.08±0.01	0.16±0.02
7MeG*	0.13±0.05	0.24±0.03	0.38±0.08	0.17±0.05

*Corrected for 80% loss during analysis (see Appendix for details).

DISCUSSION

A comparison of the ribosomes from M. hominis and E. coli revealed some differences between the two organisms however, no gross simplifications were apparent. The results presented in Figures 1 and 2 show that the sedimentation properties of ribosomes from M. hominis are similar to those of other prokaryotes when examined in the presence of 0.5-1.0 mM Mg^{2+}. The data in Table 3 indicating mycoplasma ribosomes are composed of approximately 60% RNA and 40% protein provide additional evidence that these particles fit into the general pattern of source, size, and composition characteristic of bacterial ribosomes (Spirin and Gavrilova, 1969). These findings are in general agreement with those of Kirk and Morowitz (1969) who have examined these properties for ribosomes from another species of mycoplasma, M. gallisepticum.

In contrast to these similarities, Table 2 shows that when sedimentation characteristics of 0.5 M NH_4Cl washed ribosomes from M. hominis and E. coli are compared over a wide range of Mg^{2+} concentrations, 0.05-100 mM, several differences between the two became apparent. The mycoplasma required higher concentrations of Mg^{2+} to bring about association of the subunits than did the E. coli preparations. Also, ribosomal subunits from M. hominis seemed to lose their structural integrity, judged from a shift of material sedimenting at 30-45S to 17S and 23S peaks, at Mg^{2+} concentrations in which the E. coli subunits showed no such shift. This sharp decrease in sedimentation rate may be due to an unfolding of the compact structure of the M. hominis ribosomes as has been observed with E. coli ribosomes

depleted of Mg^{2+} (Weller, et al., 1968). There were also differences noted in the effect of Mg^{2+} when M. hominis ribosomes prepared with and without a 0.5 M NH_4Cl wash were compared (Table 2). The high ionic strength wash resulted in an increase in the Mg^{2+} concentrations required both to stabilize the subunits and to effect their association.

Characterizations of the ribosomal proteins from a variety of organisms, both prokaryotic and eukaryotic, have all indicated the presence of a large number of structurally diverse proteins (Hardy, et al., 1969 and others cited therein). Since M. hominis appears to be a genetically limited organism, it was of interest to examine its ribosomal proteins to determine whether the number of components present was substantially lower than that observed in E. coli and other organisms. A comparison of the proteins from the 30S and 50S subunits of M. hominis and E. coli ribosomes by electrophoresis in polyacrylamide gels (Figure 3) gives no indication of any reduction in the complexity of the protein banding pattern. This observation is consistent with the conclusion that, at least in the case of 30S E. coli ribosomes, essentially all of the proteins are necessary for the function of the organelle (Nomura, et al., 1969).

High molecular weight rRNA of M. hominis was found to differ from that of E. coli in both its sedimentation properties and base composition. When centrifuged through sucrose density gradients, the large rRNA component from mycoplasma sedimented slightly slower than that of E. coli (Figure 4); an observation also made by Kirk and Morowitz (1969) with M. gallisepticum rRNA. However, the high levels of endogenous RNase found in Mycoplasma (Pollack, et al., 1965) suggested the

possibility that this might be an artifact of isolation. The demonstration that E. coli rRNA remains undamaged when mixed with a crude mycoplasma cell lysate argues against this possibility and indicates that the difference in sedimentation behavior between the larger rRNA species of E. coli and Mycoplasma is real. The smaller species of rRNA from the two organisms were indistinguishable by sucrose gradient centrifugation.

Gel filtration of the total RNA isolated from M. hominis revealed a fraction eluting between high molecular weight rRNA and tRNA (Figure 5). This is the position at which 5S RNA is expected to elute and rechromatography of this material with ^{14}C-labeled E. coli 5S RNA shows that the two chromatograph together on Sephadex G-100 (Figure 6). A further indication that this fraction from mycoplasma corresponds to the 5S RNA of E. coli was the complete lack of minor nucleosides noted when this RNA was analyzed by the method of Randerath, et al. (1969) (Table 4). It is known that the 5S RNA of E. coli contains no unusual nucleosides (Brownlee, et al., 1968). Assuming that the molecular weights of mycoplasma and E. coli rRNA and 5S RNA are similar, the amount of 5S RNA recovered is equivalent to 0.9 moles 5S RNA per mole of high molecular weight rRNA (16S + 23S). This is in good agreement with the finding of one molecule of 5S RNA per 50S ribosome (Rosset, et al., 1964). Mycoplasma 5S RNA was eluted from MAK columns by salt concentrations quite a bit higher than those required to elute E. coli 5S RNA or tRNA (Figure 7). This difference may be at least in part due to the lower G + C content of M. hominis 5S RNA (Table 4). It has been shown that separation on MAK columns is dependent on base composition and that RNA with

higher G + C content is eluted earlier (Sueoka and Cheng, 1967). 5S RNA has been assumed to be a universal component of ribosomes, however the experiments of Lizardi and Luck (1971) showing an apparent lack of 5S RNA in mitochondrial ribosomes makes this assumption no longer tenable. It is then worthwhile to note that the identification of 5S RNA in extracts of M. hominis represents the first report of this RNA species in mycoplasma.

Transfer RNA from M. hominis was found to have sedimentation and gel filtration properties identical to those of E. coli tRNA (Figure 8) indicating that this species of RNA from the two organisms is about the same size. The similarity of the tRNA from the two organisms is also evidenced by the ability of aminoacyl-tRNA ligases from E. coli to charge the tRNA from M. hominis (Figure 9). These results are in agreement with those of Hayashi, et al. (1969) for tRNA from M. gallisepticum, M. laidlawii, and M. sp. (Kid). A much smaller species of RNA, presumably tRNA, sedimenting at 2.5S which had been reported in M. gallisepticum (Kirk and Morowitz, 1969) was not observed in M. hominis (Figure 4 and 8b). The base composition of the tRNA was determined by two independent methods and the results are in good agreement (Table 4). The G + C content of M. hominis tRNA was found to be lower than that of E. coli tRNA. This is in keeping with the general observation (Tables 4 and 7) that all forms of mycoplasma RNA have a low G + C content, perhaps reflecting the exceptionally low level of these constituents in mycoplasma DNA (Kelton and Mandel, 1969).

The presence of unusually small amounts of modified nucleotides in the RNA from mycoplasma has been reported by several investigators (Hall,

et al., 1967; Hayashi, et al., 1969; Johnson, et al., 1970); however, see Feldmann and Falter (1971). The levels of a number of these components in the tRNA and rRNA from M. hominis were determined by extending the very sensitive technique of Randerath, et al. (1969) for use with the minor components of RNA (see Appendix). The results indicate that the levels of several modified pyrimidines are indeed considerable lower in mycoplasma tRNA than in E. coli tRNA (Table 5). The most striking difference is seen in the amount of rT in the M. hominis tRNA; it is below the limits of detection of the analytical technique, about 0.01 mole %. This represents less than one rT residue per 100 tRNA molecules. The tRNA from Mycoplasma Sp. (Kid) also lacks rT or contains only a very small amount of it (Johnson, et al., 1970). The tRNA from all other organisms which have been studied contains rT. In all tRNA molecules that have been sequenced, this base appears at a specific position in the molecule, 23 nucleotides removed from the 3' terminus, as part of a constant sequence, $G_prT_p\Psi_pC$, (Zachau, 1969). Because of its widespread occurrence, this tetranucleotide sequence is thought to play an important role in the reactions of tRNA. However, investigations by Johnson, et al. (1970) exploiting the rT deficiency of M. sp. (Kid) tRNA and also those of Svensson, et al. (1971) using tRNA from a recently isolated mutant of E. coli which also lacks rT (Björk and Isaksson, 1970) did not reveal any differences in the rate or extent of aminoacylation or the ability to participate in ribosome directed polypeptide synthesis between normal tRNA and that without rT. It is possible that the integrity of the sequence $G_prT_p\Psi_pC$ is required for a function of tRNA that has not yet been identified.

If the absence of rT is found to be a common feature of the mycoplasmas, it could provide a useful criterion for differentiating these organisms from the L-phase variants of bacteria. Since a recent report by Feldmann and Falter (1971) shows that the tRNA from M. laidlawii contains reasonably high levels of rT and other minor constituents, the validity of this criterion as a classification determinant will depend on the outcome of a recent proposal to change the taxonomic status of M. laidlawii (Edward and Freundt, 1969), as well as the investigation of this feature in other species of mycoplasma.

The levels of Ψ, H_2U, and 4TU in M. hominis tRNA were found to be about one-half those in E. coli tRNA. If these reduced levels of modified bases are due to differences in the specificity of the RNA modifying enzymes from the two organisms, the mycoplasma RNA may prove to be a useful tool for studying the biosynthesis of these nucleotides. Several precedents for this type of study can be found in the literature. Baguley, et al. (1970) found that a methylase preparation from rat liver was capable of forming a 1MeA residue at a specific position in a species of yeast tRNA. The adenosine residue methylated by the rat liver enzyme is not modified by yeast methylases. Investigations by Johnson, et al. (1970) and Bartz, et al. (1970) have shown that the tRNA from Mycoplasma sp. (Kid) can serve as a substrate for enzymes from E. coli which catalyze the formation of rT and IPA, nucleosides which this RNA lacks in vivo (Hayashi, et al. (1969). Investigations such as this would be of special interest in the cases of H_2U and Ψ since they are present in relatively low levels in the tRNA from M. hominis and there is no

unequivocal evidence regarding the mechanism of their formation in tRNA (Söll, 1971).

In contrast to the results of other investigators who have examined the minor base content of mycoplasma tRNA (Hall, et al. 1967 and Hayashi, et al. 1969), the presence of a variety of methylated purine derivatives was noted at levels which, within experimental error, equaled or exceeded those in E. coli tRNA (see also Feldmann and Falter, 1971).

Base composition analysis of the rRNA from M. hominis shows that there is a general reduction in the modified nucleoside content in the 23S RNA (0.58 mole % of the bases are modified as compared to 1.12 mole % in E. coli 23S RNA) and a slightly elevated extent of modification is observed in the 16S RNA of M. hominis when compared to that found in E. coli (1.02 and 0.85 mole % respectively). The levels of modified pyrimidines in M. hominis rRNA are within the standard deviations, always less than or equal to those found in the corresponding E. coli rRNA. No such pattern was evident with regard to the minor purine components of the rRNA from the two organisms. In view of the evidence indicating that in E. coli different enzymes form rT in tRNA and rRNA (Björk and Isaksson, 1970), it was of interest to note that both the rRNA and tRNA from M. hominis were devoid of this nucleoside. This observation indicates that M. hominis totally lacks the ability to synthesis this constituent of RNA.

The components of the protein synthesizing apparatus from M. hominis that were characterized in this investigation, the ribosomes and tRNA, were found to be somewhat different from those of E. coli however, no

extensive reductions in complexity were noted. The possibility that structural simplifications exist in other elements of this apparatus such as the aminoacyl-tRNA ligases or other soluble factors was not pursued. M. hominis ribosomes were found to contain as many or more unique protein and RNA components as other bacterial ribosomes. The slight differences observed in the size and structure of some of these components from the mycoplasma, when compared to the more extensively studied E. coli, presented no obvious advantages for the study of structure-function relationships in ribosomes. The tRNA from M. hominis was also shown to be quite similar to that of other organisms with respect to its size and shape but, it was found to contain an unusually small amount of a number of modified pyrimidine nucleosides normally found in tRNA. The low levels of Ψ, H_2U, 4TU, and especially rT in this tRNA constitute a significant simplification in the structure of tRNA and, as such, represents a potentially useful tool for studying the biosynthesis of these nucleosides and the roles they play in the function of tRNA.

SUMMARY

The ribonucleic acids and ribosomes from M. hominis strain 4330 have been characterized as part of a study to determine whether the protein synthesizing apparatus of an organism with a limited genome is significantly less complex than that of the more extensively studied prokaryotes such as E. coli.

Ribosomes from M. hominis sediment at 71S. They are composed of 61% RNA and 39% protein. These ribosomes can be dissociated into subunits with sedimentation constants of 33S and 53S in buffers containing 0.5-1.0 mM Mg^{2+}. If the Mg^{2+} concentration is reduced to less than 0.5 mM, these subunits appear to lose their structural integrity as judged by a dramatic decrease in their sedimentation coefficients. The proteins associated with the 33S and 53S subunits of mycoplasma were compared to those from the 30S and 50S subunits of E. coli ribosomes by polyacrylamide gel electrophoresis at both pH 4.5 and 8.7. In all cases, the banding patterns seen with the mycoplasma ribosomal protein preparations contained as many or more components than did the corresponding material from E. coli. This indicates that, at the level of resolution afforded by acrylamide gel electrophoresis, the protein complement of M. hominis ribosomes is at least as complex as that of E. coli.

Two species of high molecular weight RNA were isolated from crude cell extracts of M. hominis. They had sedimentation coefficients of 22S and 16S; the larger component sedimenting slightly slower than 23S RNA from E. coli in sucrose density gradients. Base composition analysis of the high molecular weight RNA from M. hominis indicated a G + C

content 46.4% as compared to 54.0% for rRNA from E. coli.

A species of RNA equal in size to E. coli 5S RNA was isolated from mycoplasma extracts by gel filtration. This RNA differed from E. coli 5S RNA in G + C content and also in its chromatographic properties on columns of methylated albumin-kieselguhr. No modified or unusual nucleosides were detected in this fraction. The molar ratio of this form of RNA to the high molecular weight rRNA was approximately 0.9:1.

Transfer RNA from M. hominis was found to have sedimentation and gel filtration properties identical to E. coli tRNA and could be charged with amino acids by the aminoacyl-tRNA ligases of E. coli.

Analyses of the minor nucleosides present indicated that M. hominis tRNA contains about one-half the amounts of dihydrouridine, pseudouridine, and 4-thiouridine and less than 1% of the ribothymidine found in E. coli tRNA.

A very sensitive method of base composition analysis involving $NaIO_4$ oxidation of nucleosides followed by reduction with [^{3}H]$NaBH_4$ was, in the course of these investigations, extended for use with a number of the modified nucleosides commonly found in RNA.

BIBLIOGRAPHY

Attardi, G., and Ojala, D. (1971), Nature New Biology 229, 133.

Baguley, B. C., Wehrli, W., and Staehelin, M. (1970), Biochem. 9, 1645.

Bartz, J. K., Kline, L. K., and Söll, D. (1970), Biochem. Biophys. Res. Commun. 40, 1481.

Beaven, G. H., Holiday, E. R., and Johnson, E. A. (1955), in The Nucleic Acids, Vol. I, Chargaff, E., and Davidson, J., Eds., New York, Academic, p 493.

Björk, G. L., and Isaksson, L. A. (1970), J. Mol. Biol. 51, 83.

Brega, A., and Vesco, C. (1971), Nature New Biology 229, 136.

Britten, R. J., and Roberts, R. B. (1960), Science 131, 32.

Broom, A. D., Townsend, L. B., Jones, J. W., and Robins, R. K. (1964), Biochem. 3, 494.

Brownlee, G. G., Sanger, F., and Barrell, B. G. (1968), J. Mol. Biol. 34, 379.

Cerutti, P., and Miller, N. (1967), J. Mol. Biol. 26, 55.

Cohn, W. E. (1960), J. Biol. Chem. 235, 1488.

Demerec, M., and Cahn, E. (1953), J. Bacteriol. 65, 27.

Dische, Z. (1955), in The Nucleic Acids, Vol. I, Chargaff, E. and Davidson, J., Eds., New York, Academic, p 301.

Edward, D. G., and Freundt, E. A. (1969), J. Gen. Microbiol. 57, 391.

Feldmann, H., and Falter, H. (1971), Europ. J. Biochem. 18, 573.

Fink, K., and Adams, W. S. (1966), J. Chromatog. 22, 118.

Fox, J. J., Pragg, D. V., Wempen, I., Doerr, I. L., Cheong, L., Knoll, J. E., Eidinoff, M. L., Bendich, A., and Brown, G. B. (1959), J. Amer. Chem. Soc. 81, 178.

Fraenkel-Conrat, H., Singer, B., and Tsugita, A. (1961), Virology 14, 54.

Gerster, J. F., and Robins, R. K. (1965), J. Amer. Chem. Soc. 87, 3752.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), J. Biol. Chem. 177, 751

Green, M., and Cohen, S. S. (1957), J. Biol. Chem. 225, 397.

Hall, R. H., Mittleman, A., Horoszewicz, J., and Grace, J. T., Jr. (1967), Ann. N. Y. Acad. Sci. 143, 799.

Hardy, S. J. S., Kurland, C. G., Voynow, P., and Mora, G. (1969), Biochem. 8, 2897.

Hayashi, H., Fisher, H., and Söll, D. (1969), Biochem. 8, 3680.

Ingle, J., and Burns, R. G. (1968), Biochem. J. 110, 605.

Janion, C., and Shugar, D. (1960), Acta Biochem. Pol. 7, 309.

Johnson, J. A., Jr., Thomas, H. J., and Schaeffer, H. J. (1958), J. Amer. Chem. Soc. 80, 699.

Johnson, J. L., Yamamoto, K. R., Weislogel, P. O., and Horowitz, J. (1969), Biochem. 8, 1901.

Johnson, L., Hayashi, H., and Söll, D. (1970), Biochem. 9, 2823.

Jones, J. W., and Robins, R. K. (1963), J. Amer. Chem. Soc. 85, 193.

Kelton, W. H., and Mandel, M. (1969), J. Gen. Microbiol. 56, 131.

Kinard, F. E. (1957), Rev. Sci. Instr. 28, 293.

Kirk, R. G., and Morowitz, H. J. (1969), Amer. J. Vet. Res. 30, 287.

Kurland, C. G. (1960), J. Mol. Biol. 2, 83.

Lane, B. G. (1963), Biochim. Biophys. Acta 72, 110.

Leboy, P. S., Cox, E. C., and Flaks, J. G. (1964), Proc. Natl. Acad. Sci. U.S. 52, 1367.

Lengyel, P., and Söll, D. (1969), Bacteriol. Rev. 33, 264.

Lipsett, M. N. (1966), Cold Spring Harbor Symp. Quant. Biol. 31, 449.

Lizardi, P. M., and Luck, D. J. L. (1971), Nature New Biology 229, 140.

Loening, U. E. (1967), Biochem. J. 102, 251.

Lowrie, R. J., and Bergquist, P. L. (1968), Biochem. 7, 1761.

Lowry, O. H., Rosebrough, M. J., Farr, A. L., and Randall, R. S. (1951), J. Biol. Chem. 193, 265.

Mandel, H. G., (1970) in Prog. in Mol. and Subcellular Biol., Vol. I, Hahn, H. T., Puck, T. T., Springer, G. F., and Wallenfels, K., Eds., New York, Springer-Verlag, p 82.

Mandell, J. D., and Hershey, A. D. (1960), Anal. Biochem. 1, 66.

Maniloff, J. Morowitz, H. J., and Barrnett, R. J. (1965), J. Bacteriol. 90, 193.

Maniloff, J. (1969), J. Bacteriol. 100, 1402.

Martin, R. G., and Ames, B. N. (1961), J. Biol. Chem. 236, 1372.

Midgley, J. E. M. (1962), Biochim. Biophys. Acta 61, 513.

Morowitz, H. J. (1966), in Ciba Foundation Symp., Principals of Biomolecular Organization, Wolstenholme, G. E. W., and O'Conner, M., Eds., Boston, Little-Brown and Co., p 446.

Morowitz, H. J., Bode, H. R., and Kirk, R. G. (1967), Ann. N.Y. Acad. Sci. 143, 110.

Muench, K. H., and Berg, P. (1966) in Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Eds., New York, Harper and Row, p 375.

Nomura, M., Mizushima, S., Ozaki, M., Traub, P. and Lowry, C. V. (1969), Cold Spring Harbor Symp. Quant. Biol. 34, 49.

Pene, J. J., Knight, E., Jr., and Darnell, J. E., Jr. (1968), J. Mol. Biol. 33, 609.

Pollack, J. D., Razin, S., and Cleverdon, R. C. (1965), J. Bacteriol. 90, 617.

Randerath, E., Ten Broeke, J. W., and Randerath, K. (1968), FEBS Letters 2, 10.

Randerath, K. (1969), Anal. Chem. 41, 991.

Randerath, K. (1970), Anal. Biochem. 34, 188.

Randerath, K., Flood, K. M., and Randerath, E. (1969), FEBS Letters 5, 31.

Randerath, K. and Randerath, E. (1969), Anal. Biochem. 28, 110.

Randerath, K. and Randerath, E. (1971), in Proc. in Nucleic Acid Res., Vol. II, Cantoni, G. L., and Davies, D. R., Eds., New York, Harper and Row, p 796. (in press)

Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), Nature 195, 281.

Robins, M. J., Hall, R. H., and Thedford, R. (1967), Biochem. 6, 1837.

Rodwell, A. (1969), in The Mycoplasmatales and the L-Phase of Bacteria, Hayflick, L., Ed., New York, Appleton-Century-Crofts, p 413.

Rosset, R., Monier, R., and Julien, J. (1964), Bull. Soc. Chim. Biol. 46, 87.

Sarin, P. S., and Zamecnik, P. C. (1964), Biochim. Biophys. Acta 91, 653.

Schleich, T., and Goldstein, J. (1966), J. Mol. Biol. 15, 136.

Smith, J. D., and Dunn, D. B., (1959), Biochem. J. 72, 294.

Söll, D. (1971), Science 173, 293.

Solymosy, F., Fedorcsak, I., Gulyas, A., Farkas, G. L., and Ehrenberg, L. (1968), Europ. J. Biochem. 5, 520.

Spirin, A. S., and Gavrilova, L. P. (1969), The Ribosome, New York, Springer-Verlag, p 1.

Spitnik-Elson, P. (1965), Biochem. Biophys. Res. Commun. 18, 557.

Stanley, W. M., Jr. and Bock, R. M. (1965), Biochem. 4, 1302.

Starr, J. L., and Sells, B. H. (1969), Physiol. Rev. 49, 623.

Sueoka, N. and Cheng, T. (1967), in Methods in Enzymology, Vol. XII, Part A, Colowick, S. P. and Kaplan, N. O., Eds., New York, Academic, p 562.

Svensson, I., Isaksson, L., and Henningsson, R. (1971), Biochim. Biophys. Acta 238, 331.

Thedford, R., Fleysher, M. H., and Hall, R. H. (1965), J. Med. Chem. 8, 486.

Tourtellotte, M. E. (1969), in The Mycoplasmatales and the L-Phase of Bacteria, Hayflick, L., Ed., New York, Appleton-Century-Crofts, p 451.

Townsend, L. B., Robins, R. K., Loeppky, R. N., and Leonard, N. J. (1964), J. Amer. Chem. Soc. 86, 5320.

Traut, R. R., Delius, H., Ahmad-Zadeh, C., Bickle, T. A., Pearson, P., and Tissieres, A. (1969), Cold Spring Harbor Symp. Quant. Biol. 34, 25.

Wacker, V. A., and Ebert, M. (1959), Z. Naturforsh. 14B, 709.

Weller, D., Schecter, Y., Musgrave, D., Rougvie, M., and Horowitz, J. (1968), Biochem. 7, 3668.

Yung, N. C., Burchenal, J. H., Fecher, R., Duschinsky, R., and Fox, J. J. (1961), J. Amer. Chem. Soc. 83, 4060.

Zachau, H. G. (1969), Angew. Chem. 81, 645.

Zachau, H. G., Dütting, D., Feldman, H. (1966), Angew. Chem. 78, 392.

Ziff, E. B., and Fresco, J. R. (1968), J. Amer. Chem. Soc. 90, 7338.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. Jack Horowitz for his guidance and encouragement throughout this investigation. The time, ideas, and efforts contributed by fellow graduate students is also gratefully acknowledged.

A special thanks is due the author's wife, Germaine, who, through her patience, encouragement, and unselfish love, contributed immeasurably to the completion of this work.

The author also wishes to acknowledge the U. S. Public Health Service National Institutes of Health for financial assistance through a Predoctoral Fellowship to the author (2-F01-GM-42, 211-03) and a research grant (GM-09042) to Professor Horowitz.

APPENDIX:

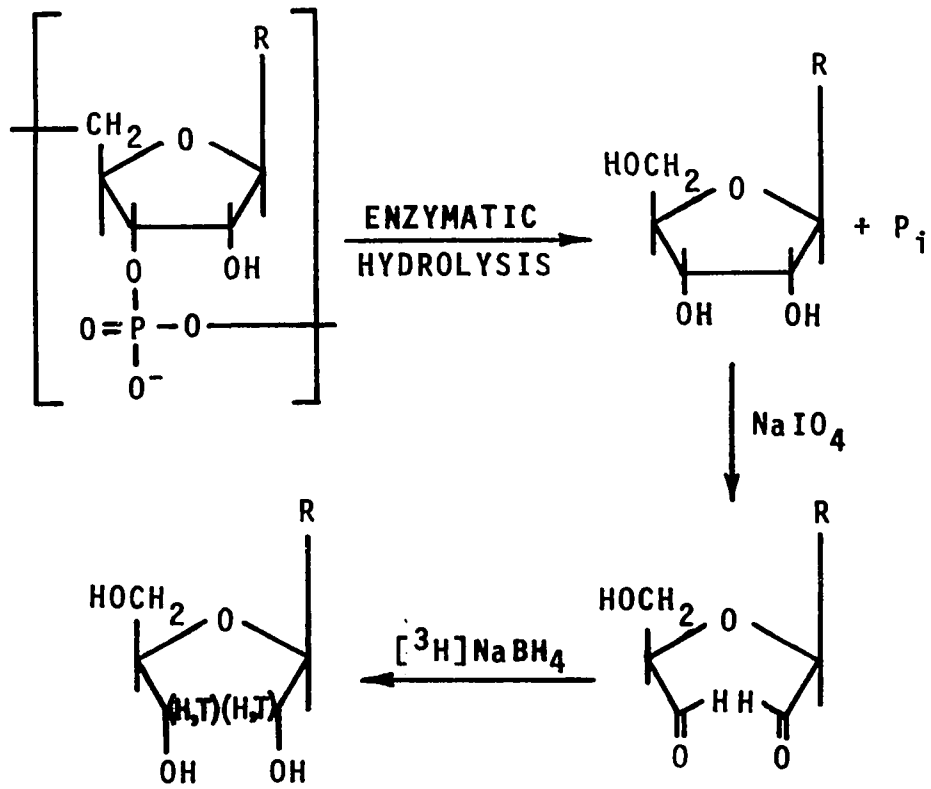
DETERMINATION OF LOW LEVELS OF MODIFIED NUCLEOSIDES IN RNA

The identification and characterization of RNA which either lacks or has an extremely low level of minor components requires an analytical technique capable of detecting and accurately measuring very small amounts of these components in the presence of a large excess of the major nucleosides. A method involving the reduction of $NaIO_4$ oxidized nucleosides with high specific activity $[^3H]NaBH_4$ developed by Randerath et al. (1968) and Randerath and Randerath (1969) seemed to be well suited to our needs. A summary of the reactions involved are shown in Figure 10. The RNA is enzymatically hydrolyzed to its constituent nucleosides and, the resulting nucleosides are then oxidized to dialdehyde derivatives with $NaIO_4$. Reduction with $[^3H]NaBH_4$ yields trialcohol derivatives of the nucleosides which, after being treated with H_2O to remove exchangeable tritium, contain 2 moles of tritium per mole of nucleoside. This technique was known to give quantitative results with the four major nucleosides (Randerath and Randerath, 1969), and a method for the separation of a large number of the modified nucleosides commonly found in RNA had been developed (Randerath, et al., 1969). A detailed description of our adaptation of this technique, and its extension to the quantitative measurement of a number of modified nucleosides follows.

Hydrolysis

RNA samples to be analyzed were dialyzed extensively against H_2O before being hydrolyzed to remove any substances, such as Tris or sucrose,

Figure 10. Schematic representation of the reactions used in the ^{3}H-labeling method of base composition analysis.


R
CH2
O
OH
O=P–O
O⁻
ENZYMATIC
HYDROLYSIS
HOCH2
+ Pi
NaIO4
[3H]NaBH4
HH
(H,T)(H,T)

which interfere by reacting with $NaIO_4$ and/or [^{3}H]$NaBH_4$. The RNA was then hydrolyzed in a mixture made up by combining: 2 µl of 0.1 M $MgCl_2$, 1 µl of 0.6 M Bicine (pH 8.0), 2 µl of an enzyme mixture containing 2.0-2.5 µg/ml each of snake venom phosphodiesterase, pancreatic RNase, and bacterial alkaline phosphatase (dialyzed against H_2O to remove $(NH_4)_2SO_4$), 15-25 µg of RNA, and H_2O to bring the final volume to 20 µl. This mixture was incubated at 37° for 22-24 h in a tightly sealed tube, 2 cm x 0.6 cm. A blank containing no RNA was treated identically and carried through all of the following steps.

Oxidation-Reduction-H_2O Exchange

2 µl Aliquots were removed from the hydrolysis mixture and diluted with 15 µl of H_2O. 2 or 3 µl of a freshly prepared solution of $5 \cdot 10^{-3}$ M $NaIO_4$, enough to maintain approximately a two fold molar excess of periodate over nucleosides, were then added and the resulting solution incubated for 2 h at 25° in the dark. The resulting nucleoside dialdehydes were reduced by adding 1 µl of 0.1 M [^{3}H]$NaBH_4$ (2.08 Ci/mM) in 0.1 M NaOH. It is important to maintain at least a five fold molar excess of [^{3}H]$NaBH_4$ over $NaIO_4$ to avoid the formation of nucleoside monoaldehydes (Randerath and Randerath, 1971). [^{3}H]$NaBH_4$ solutions of this specific activity were stored in small aliquots at -85° for 6 months with no detectable loss of reducing power. Reduction was allowed to proceed for 2 h at 25° in the dark. This reaction was stopped by the addition of 25 µl of 1.0 M acetic acid to the reduction mixture; this also destroyed excess sodium borotritiide. Because $^3H_2$ is liberated at this step, the operation must be carried out in a well ventilated

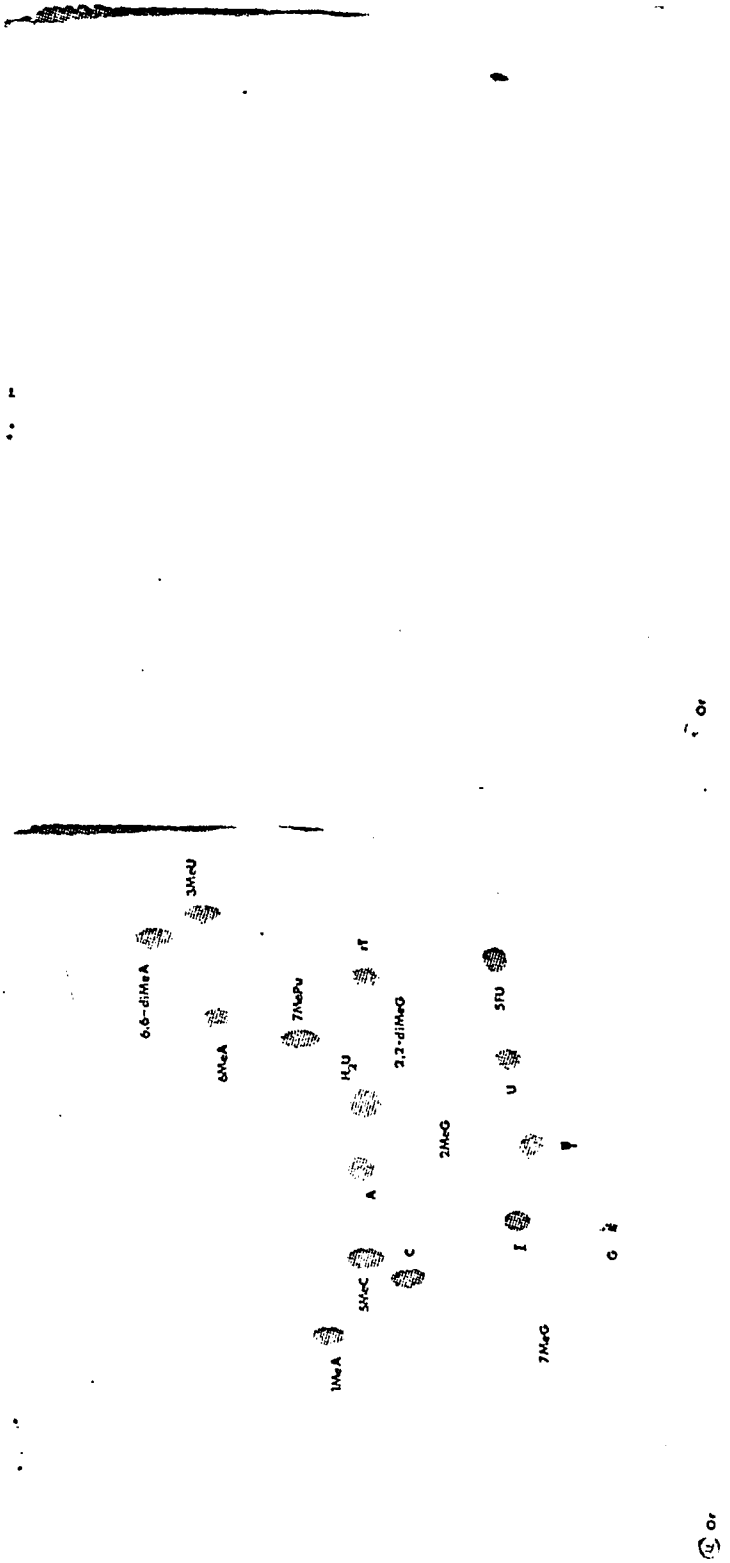
hood. The mixture was allowed to stand at room temperature for 15 min and then evaporated to dryness in a vacuum desiccator. To remove exchangeable tritium, the residue was treated three times with 50 µl portions of H_2O and dried each time in a vacuum desiccator as before.

Thin-Layer Chromatography

The residue, after the third exchange with H_2O was taken up in 10 µl of 0.1 M HCO_2H and spotted in 2 µl aliquots on a 20 cm x 20 cm precoated, plastic-backed, cellulose TLC sheet (Eastman Organic Chem. #6064). The reaction tube was then rinsed with 10 µl of 0.1 M HCO_2H which was also spotted on the TLC sheet. If Brinkman MN-POLYGRAM thin-layer chromatography sheets were substituted for those from Eastman Organic Chem., the radioactive spots became very diffuse resulting in a loss of resolution for a number of nucleosides.

The chromatograms were developed once in the first dimension in a solvent consisting of 1-butanol, 2-propanol, 7.5 M NH_4OH (3:3:2). The chromatograms, after thorough air drying, were then developed in the second dimension in a solvent consisting of 2-butanone, 2-methyl-2-butanol, H_2O, 23.5 M HCO_2H (2:2:1:0.1). After thorough drying, the TLC plates were again developed in the second dimension using the same solvent. To achieve a useful separation of 2,2-diMeG from H_2U, it was necessary to allow the second solvent to run the entire width of the chromatogram during both irrigations. Also, individual spots were sharper if the atmosphere in the tank was not saturated with solvent vapors. A map of the nucleosides which were examined in this study is shown in Figure 11. 1MeG migrates to the same position as A in this solvent system and hence could not be measured in RNA hydrolysates.

Figure 11. The fluorograph of a cellulose TLC plate showing the chromatographic behavior of 2',3'-^{3}H-trialcohol derivatives of the nucleosides analyzed in this investigation. The right side of the figure shows a blank from which all nucleosides have been omitted. Each spot represents the radioactivity from 50-100 picomoles of the nucleoside with the exception of 2MeG which represents 10 picomoles.


3MeU
6,6-diMeA
6MeA
7MePu
rT
H2U
2,2-diMeG
5FU
U
2MeG
A
C
5MeC
I
1MeA
7MeG

A new solvent system which affords better resolution of 1MeG, A, H_2U and 2,2-diMeG has recently been described by Randerath and Randerath (1971).

Fluorography

Conventional autoradiographic techniques which are useful for detecting β-emitters such as ^{14}C and ^{32}P are too insensitive to ^{3}H to be useful because of the low energy and short range of the β-emission from this isotope. To overcome this problem, the ^{3}H containing chromatograms were impregnated with 2,5-diphenyloxazole which can convert the ^{3}H-β-particles to light that then produces an image on X-ray film. The details of this technique have been described by Randerath (1969; 1970). Chromatograms were coated by rapidly pouring 15-16 ml of a 7% solution of 2,5-diphenyloxazole in diethyl ether over each 20 cm x 20 cm TLC sheet; excess solution is allowed to run off an edge of the chromatogram. To obtain a uniform covering of the entire sheet, this operation must be carried out very quickly. Working in a photographic darkroom, each chromatogram was stapled to a sheet of X-ray film. This film-TLC packet was inserted in a redi-pak container (Eastman Kodak), taped to exclude all light, and then pressed between two sheets of glass which were held tightly together with screw clamps. Exposure of the film was carried out at -78.5^{o} by packing dry ice around the film-TLC sheet sandwich. Exposure times varied depending on the amount of ^{3}H present on the TLC sheet. Spots containing 7-10 nanocuries of $^{3}H/cm^{2}$ could be visualized after an exposure of one day. Each spot in Figure 11 represents the radioactivity from 50-100 picomoles of each nucleoside with the exception of 2MeG which represents 10 picomoles.

Quantitation of Nucleosides

The developed film was repositioned over the chromatogram using the staple holes as markers. The positions of the radioactive compounds were marked on the film image and the underlying chromatogram with needle holes. The marked spots on the TLC sheets were then cut out with scissors and the radioactive nucleoside trialcohols eluted with H_2O. Spots representing the major nucleosides contained large amounts of radioactivity and were eluted with 5 ml of H_2O while the minor components were eluted with 1.0 or 3.0 ml. Elution was carried out by shaking these suspensions for a minimum of 12 h. 0.5 Ml of the eluate from each spot was then counted in a liquid scintillation spectrometer using the scintillation cocktail developed by Kinard (1957) modified by the addition of Cab-O-Sil as described by Randerath and Randerath (1969). The ^3H counting efficiency of this system was 25-30%.

Results

To reaffirm the quantitative aspects of this technique with the major components of RNA, G, C, A, and U, results from analyses of synthetic mixtures of these nucleosides and also of unfractioned E. coli tRNA were compared with spectrophotometric analyses of the same samples. Table 8 shows the results of these comparisons. The excellent agreement between the two methods confirms the results of Randerath, et al. (1968).

Quantitation of modified nucleosides was done as follows. A sample of the commercial preparations of each compound was subjected to a complete analysis in the absence of all other nucleosides to confirm

Table 8. Comparison of spectrophotometric and ^{3}H-incorporation methods for the analysis of G, C, A, and U in synthetic mixtures of nucleosides and tRNA

Nucleoside	Mole % in: Nucleoside Mixtures			tRNA		
	Spectro.	^{3}H	$\frac{^{3}H}{\text{Spectro.}}$ (x100)	Spectro.	^{3}H	$\frac{^{3}H}{\text{Spectro.}}$ (x100)
G	18.9	18.8	99	29.5	29.3	99
C	24.4	24.2	99	26.7	27.1	101
A	21.7	20.7	95	21.3	21.1	99
U	35.0	36.1	103	22.7	22.4	99

the chromatographic behavior of the 2',3'-^{3}H-trialcohol derivative (Randerath, et al., 1969), and also to check the purity of the preparation. The samples of Ψ, H_2U, 1MeA, 6MeA, and IPA all showed multiple components when analyzed by the ^{3}H-incorporation method and were purified by either paper or thin-layer chromatography before being used for quantitative determinations (Fink and Adams, 1966; Green and Cohen, 1957; Robins, et al., 1967). A sufficient purification of all the nucleosides was achieved by these methods with the exception of IPA which will be discussed later. Stock solutions of these nucleosides were then prepared and analyzed by the ^{3}H-labeling method. A standard amount of guanosine was included in each mixture to serve as a reference and the amount of tritium found in each spot was expressed relative to that found for guanosine. These ratios were then compared to the same number determined spectrophotometrically. The results are presented in Table 9; a value of 1.00 indicates complete agreement between the two analyses.

The nucleosides listed in the first column of Table 9 all show excellent agreement between the two methods.

The second column indicates those nucleosides for which the ratio deviated appreciable from 1.00. There was no anomalous behavior such as degradations or rearrangements observed with any of these compounds. The most likely source of error in these determinations seemed to be the molar extinction coefficients. Extinction coefficients varying by up to 10% were found for a number of nucleosides (compare values for 2MeG given by Gerster and Robins (1965) with that of Smith and Dunn (1959)). The values used in the analyses summarized in Table 9 are listed in Table 1. These numbers were chosen from the literature on the basis

Table 9. Solutions containing spectrophotometrically determined amounts of several modified nucleosides were analyzed by the ^3H-incorporation method. The base compositions determined by each method are compared as a ratio; a value of 1.00 indicates complete agreement between the two methods

Nucleoside	$\frac{^3H}{Spectro.}$	Nucleoside	$\frac{^3H}{Spectro.}$	Nucleoside	$\frac{^3H}{Spectro.}$
Ψ	1.00±0.02	I	1.11±0.07	7MeG	0.19±0.02
H_2U	1.00±0.07	6MeA	0.90±0.04	1MeA	0.72±0.02
3MeU	1.07±0.07	6,6-diMeA	0.91±0.03		
5FU	1.03±0.05	2MeG	1.18±0.04		
5MeC	1.04±0.03	2,2-diMeG	0.86±0.07		
		1MeG	0.94±0.10		

of the apparent quality of their determination rather than any expendiency. It can be seen from the example of 2MeG given above that these uncertainties in molar extinction coefficients can account for many of the deviations seen in column two of Table 9.

Column three of Table 9 contains the nucleosides which were altered in the course of the analysis but could still be reliably estimated by applying correction factors. The data in column three which was used to determine these factors is the average of six determinations of each nucleoside. 7MeG is lost largely during the prolonged incubation at pH 8.0 necessary for the complete conversion of the RNA to nucleosides. Some losses also occur during the reduction with [^{3}H]$NaBH_4$ which takes place at an elevated pH (Randerath and Randerath, 1971). The alkaline conditions just mentioned also cause the rearrangement of 1MeA to 6MeA. Experiments with purified nucleosides showed that 33% of the 1MeA present initially is isomerized to 6MeA under the conditions employed in these analyses. As a result of these losses, base composition data for RNA samples were corrected as follows: by a factor of 5 for the 7MeG content; by a factor of 1.5 for the 1MeA content; and the 6MeA level was reduced by an amount equal to one-third of the corrected 1MeA value.

Discussion

The results presented in Tables 8 and 9 show that the ^{3}H-incorporation method of base composition analysis is useful for the quantitative determination of a large number of the nucleosides found in RNA. The capacity of this technique to measure RNA constituents present in very

small amounts is limited by the specific activity of [^{3}H]$NaBH_4$ and the sensitivity of fluorographic detection. Using [^{3}H]$NaBH_4$ with a specific activity of 2 Ci/mM, the digest from 5 μg of RNA would yield about 15 μCi of radioactive nucleoside derivatives. Since the fluorography has a sensitivity limit of about 10 nanocuries/cm^2/day, by exposing the X-ray film for 2 days, nucleosides present at the level of 1 base in 3000 could be detected. By using [^{3}H]$NaBH_4$ with a specific activity of 15-20 Ci/mM (commercially available) this sensitivity could be increased by about an order of magnitude with no increase in either exposure time or the amount of RNA. The only base composition analysis method which exceeds this sensitivity involves in vivo labeling of the RNA with high specific activity ^{32}P and is therefore limited to systems where this isotope can be administered (Midgley, 1962).

There are some modified nucleosides which are known to occur in RNA that can not be measured using this technique. 2'-O-methylnucleosides are not reactive with $NaIO_4$ and therefore do not incorporate tritium when treated with [^{3}H]$NaBH_4$. 4TU is oxidized by $NaIO_4$ to a sulfonic acid derivative that is subsequently converted to uridine (Ziff and Fresco, 1968). An attempt to measure IPA, a nucleoside which occurs adjacent to the anticodon in some species of tRNA (Zachau, et al., 1966), by the ^{3}H-incorporation method was not successful. Although this nucleoside could be purified to apparent homogeneity by paper chromatography using the solvent systems of Robins, et al., (1967), when these preparations were analyzed by the ^{3}H-labeling method, a second spot containing about one-half the radioactivity of the IPA locus was always present. Also, if IPA that showed only a

single component (R_f = 0.84-0.87) on paper chromatograms developed in 2-butanone, 2-methyl-2-butanol, H_2O, 23.5 M HCO_2H (2:2:1:0.1) was subjected to the hydrolysis step used in RNA analyses and then rechromatographed in this same solvent, a second component which trailed back to the origin (R_f = 0-0.13) appeared. Finally, the fraction of IPA recovered from tritium labeling analyses was too variable (49%±11%) to allow a reliable correction factor to be determined. A spot appearing on the fluorograms, designated 7MePu in Figure 11, contains radioactivity, probably as ^{3}H-glycerol (Randerath and Randerath, 1971) derived largely from purine components methylated in the 7 position. ^{3}H-glycerol from the overoxidation of other nucleosides also contributes to this spot (Randerath and Randerath, 1971), hence it is not useful for quantitative measurements of any RNA constituents. 1MeG coincided with A on the thin-layer chromatograms and therefore could not be measured in RNA hydrolysates. A different solvent system has recently been developed in which these two compounds can be resolved (Randerath and Randerath, 1971).

The quantitative relationship of H_2U to the other nucleosides was subject to additional investigation because of its known reactivity with [^{3}H]$NaBH_4$. Cerutti and Miller (1967) have shown that under conditions somewhat similar to those used in this analysis the pyrimidine ring of the H_2U undergoes a reductive cleavage incorporating 2 moles of tritium to form a β-ureidopropanol derivative. To test the possibility that this was occurring in the base composition determinations, analyses of H_2U were done in which the $NaIO_4$ oxidation was

omitted. Less than 0.1% of the expected radioactivity was found in these experiments indicating that the H_2U ring is not being reductively cleaved to any appreciable extent under the conditions employed for this analysis.

The demonstration that 5FU, a nucleoside analogue which can be incorporated into the RNA of many organisms (see review by Mandel, 1970) was quantitatively converted to the 2',3'-^{3}H-trialcohol derivative and completely separated from other components by the thin-layer chromatography (Table 9 and Figure 11) makes this method useful for studies of the effect of this drug on RNA structure. By applying the technique to 5FU substituted RNA from E. coli, the results of Johnson, et al. (1969) and Lowrie and Bergquist (1968) regarding the effects of 5FU on the minor bases of tRNA were confirmed. In addition, because of the high sensitivity of the ^{3}H-labeling technique, it was also possible to examine the effect of 5FU on the minor components of the rRNA where these constituents are much less abundant. These results indicated that the incorporation of 5FU into rRNA, as well as tRNA, causes a decrease in the amounts of Ψ, H_2U, and rT in these structures.

This technique is potentially useful for measuring a much greater variety of nucleosides and their analogues than were tested in these experiments. As more standards become available for testing, the list of compounds which can be quantitatively measured by this method will very likely be expanded.